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Kay L. Gaviglio
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Docket Number	GC817P	Type a plus sign (+) inside this box →	+

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TITLE OF THE INVENTION (280 characters max)

PEPTIDES AND SUPPORTED PEPTIDES FOR TREATING SKIN DISEASES

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ENCLOSED APPLICATION PARTS (*check all that apply*)

☒ Specification ☒ Drawing(s)—Figures	*Number of Pages:* 56 *Number of Sheets:* 13	☐ Other (specify)_____	

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☐ A check or money order is enclosed to cover the Provisional filing fees. ☒ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 07-1048.	PROVISIONAL FILING FEE AMOUNT ($)	$160.00

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Respectfully submitted,

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Name of person signing

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PROVISIONAL PATENT
Docket No. GC 817P

PEPTIDES AND SUPPORTED PEPTIDES FOR TREATING SKIN DISEASES

The present application is related to co-pending application Attorney Docket No. GC815P filed November 6, 2003, Express Mail Receipt No. EL 967 262 855 US, and is entitled, "Expression in Filamentous Fungi of Protease Inhibitors and Variants Thereof," which is incorporated by reference herein and to which this application claims priority.

Field of the invention

The invention is directed to peptides and supported peptides for treating proliferative diseases. Specifically, the invention is directed to peptides and supported peptides for treating diseases of the skin, such as rosacea.

Background of the invention

Angiogenesis is the development of a blood supply to a given area of tissue. Angiogenesis may be part of normal embryonic development, represent the revascularization of a wound bed or involve the stimulation of vessel growth by inflammatory or malignant cells. Angiogenesis is also the process through which tumors or inflammatory conditions derive a blood supply through the generation of microvessels.

Angiogenesis is regulated in normal and malignant cancer tissues by the balance of angiogenic stimuli and angiogenic inhibitors that are produced in the target tissue and at distant sites (Fidler et al., 1998; McNamara et al., 1998). Vascular endothelial growth factor-A (VEGF, also known as vascular permeability factor, VPF) is a primary stimulant of angiogenesis. VEGF is a multifunctional cytokine that is induced by hypoxia and oncogenic mutations and can be produced by a wide variety of tissues (Kerbel et al., 1998; Mazure et al., 1996).

The recognition of VEGF as a primary stimulus of angiogenesis in pathological conditions has led to various attempts to block VEGF activity. Inhibitory anti-VEGF receptor antibodies, soluble receptor constructs, antisense strategies, RNA aptamers against VEGF and low molecular weight VEGF receptor tyrosine kinase (RTK) inhibitors have all been proposed for use in interfering with VEGF signaling (Siemeister et al., 1998). In fact, monoclonal antibodies against VEGF have been shown to inhibit human tumor xenograft growth and ascites formation in mice (Kim et al., 1993; Asano et al., 1998; Mesiano et al., 1998; Luo et al., 1998a; 1998b; Borgstrom et al., 1996; 1998).

RTKs comprise a large family of transmembrane receptors for polypeptide growth factors with diverse biological activities. The intrinsic function of RTKs is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses. (Ullrich & Schlessinger, 1990, Cell 61:203-212).

Angiogenesis, involving VEGF and RTKs is not only involved in cancer development. Many diseases or conditions affecting different physiological systems are angiogenesis-dependent such as arthritis and atherosclerotic plaques (bone and ligaments), diabetic retinopathy, neovascular glaucoma, macular degeneration, ocular herpes, trachoma and corneal graft neovascularization (eye), psoriasis, scleroderma, rosacea, hemangioma and hypertrophic scarring (skin), vascular adhesions and angiofibroma (blood system).

VEGF is an angiogenesis factor of major importance for skin vascularization (Detmar 2000). VEGF expression is upregulated in the hyperplastic epidermis of psoriasis (Detmar, Yeo et al. 1995), in healing wounds and in other skin diseases characterized by enhanced angiogenesis (Detmar 2000). Targeted overexpression of VEGF in the epidermis of transgenic mice resulted in enhanced skin vascularization with equal numbers of tortuous and leaky blood vessels (Detmar, Brown et al., 1998). Also, chronic synthesis of VEGF in mouse skin leads to the first histologically equivalent murine model of human psoriasis (Xia et al., 2003), reversible by binding agents specific for VEGF.

The Bowman-Birk protease inhbitor (BBI) is a designation of a family of stable low molecular weight trypsin and chymotrypsin enzyme inhibitors found in soybeans and various other seeds, mainly leguminous seeds and vegetable materials. BBI is a family of disulfide bonded proteins with a molecular weight of about 8 kD. Chou et al. (1974) Proc. Natl. Acad. Sci. USA 71:1748-1752; Yavelow et al. (1985) Proc. Natl. Acad. Sci. USA

82:5395-5399; and Yavelow et al. (1983) Cancer Res. (Suppl.) 43:2454s-2459s. BBI has a pseudo-symmetrical structure of two tricyclic domains each containing an independent native binding loop, the native loops containing binding sites for both trypsin and chymotrypsin (Liener, I. E., in R. J. Summerfield and A. H. Bunting (eds), Advances in Legume Science, Royal Bot. Gardens, Kew, England). These binding sites each have a canonical loop structure, which is a motif found in a variety of serine proteinase inhibitors (Bode & Huber, Eur. J. Biochem. (1992) 204:433-451). Commonly, as in one of the soybean inhibitors, one of the native loops inhibits trypsin and the other inhibits chymotrypsin (Chen et al., J. Biol. Chem. (1992) 267:1990-1994; Werner & Wemmer, 1992; Lin et al., Eur. J. Biochem. (1993) 212:549-555; Voss et al., Eur. J. Biochem. (1996) 242:122-131) though in other organisms (e.g., Arabidopsis), both loops are specific for trypsin.

The Kunitz-type soybean trypsin inhbitor (STI) is another protease inhbitor that inhibits the proteolytic activity of trypsin by the formation of a stable stoichiometric complex. (*See*, e.g., Liu, K., Chemistry and Nutritional value of soybean components. In: Soybeans, chemistry, technology and utilization. pp. 32-35 (Aspen publishers, Inc., Gaithersburg, Md., 1999)). STI consists of 181 amino acid residues with two disulfide bridges and is roughly spherically shaped. (See, e.g., Song et al., J. Mol. Biol. 275:347-63 (1998)). The two disulfide bridges form two native binding loops similar to those described below for BBI.

Eglin C is a small monomeric protein that belongs to the potato chymotrypsin inhibitor family of serine protease inhibitors. The proteins that belong to this family are usually small (60-90 amino acid residues in length) and contain no disulfide bonds. Eglin C, however, is highly resistant to denaturation by acidification or heat regardless of the lack of disulfide bonds to help stabilize its tertiary structure. The protein occurs naturally in the leech *Hirudo medicinalis*.

Summary of the invention

The current invention relates to peptides and supported peptides. Specifically, the invention is drawn to a cosmetic or pharmaceutical compound for improving the appearance of skin. The current invention discloses, among other things, peptides that block binding of a protein, such as a VEGF, the peptide being expressed in a protease-

resistant scaffold. .The scaffold may be a protease inhibitor, such as (BBI), (STI) or Eglin chymotrypsin inhibitor.

In a first aspect, the invention is drawn to a cosmetic or pharmaceutical compound for improving the appearance of skin comprising a polypeptide or a peptide. The polypeptide or peptide may be one that binds to VEGF and blocks its downstream activity. In one embodiment, the compound is a peptide. In another embodiment, the compound is a polypeptide. In a preferred embodiment, the peptide has an amino acid sequence selected from the group consisting of SEQ ID NO:1-SEQ ID NO:7. In a preferred embodiment, the peptide has a conserved binding sequence, the sequence being XXLWPXWC. In a preferred embodiment, the sequence comprises SEQ ID NO:1. In a preferred embodiment, the compound has a sequence, the sequence being at least 70%, preferably 80%, preferably 90%, preferably 95% homologous to the sequences set forth herein. In a preferred embodiment, the polypeptide has a molecular weight that is preferably between 500 Daltons and 30,000 Daltons, preferably between 1000 Daltons and 10,000 Daltons, preferably from 1500 Daltons to 8,000 Daltons.

In a preferred embodiment, the compound is used for the improvement of skin in an organism with a skin disorder. In a preferred embodiment, the skin disorder is an angiogenic skin disorder. In a preferred embodiment, the skin disorder is at least one selected from the group consisting of psoriasis, venous ulcers, acne, rosacea, warts, eczema, hemangiomas and lymphangiogenesis, among numerous others. In a preferred embodiment, the skin disorder is rosacea.

In a second aspect, the invention is drawn to a cosmetic or pharmaceutical compound for improving the appearance of skin comprising a peptide or a polypeptide and a scaffold, the peptide or polypeptide being expressed in the scaffold, preferably the peptide or polypeptide being a loop, preferably, the loop being closed by a disulfide bond. The peptide or polypeptide may be one that binds to VEGF and blocks its downstream activity. In a preferred embodiment, the scaffold is STI, Eglin or BBI. In a preferred embodiment, the preferred scaffold is BBI.

In a preferred embodiment, the compound further comprises a peptide. Preferably, the peptide has an amino acid sequence selected from the group consisting of SEQ ID NO: 1- SEQ ID NO: 7. Preferably, the compounds comprises one of SEQ ID NO: 9- SEQ ID NO: 11. Preferably, the compound comprises SEQ ID NO:9. In a preferred embodiment,

the peptide has a conserved binding sequence, the sequence being XXLWPXWC. In a preferred embodiment, the compound has a sequence, the sequence being at least 70%, preferably 80%, preferably 90%, preferably 95% identical to the sequences set forth herein. The peptide molecular weight is preferably between 500 Daltons and 45,000 Daltons, preferably between 1000 Daltons and 12,000 Daltons, preferably from 1500 Daltons to 10,000 Daltons. In a preferred embodiment, the compound preferably comprises a polypeptide.

In a preferred embodiment, the compound is used for the improvement of skin in an organism with a skin disorder. In a preferred embodiment, the skin disorder is an angiogenic skin disorder. In a preferred embodiment, the skin disorder is at least one selected from the group consisting of psoriasis, venous ulcers, acne, rosacea, warts, eczema, hemangiomas, lymphangiogenesis, among numerous others. In a preferred embodiment, the skin disorder is rosacea.

In a third aspect, the invention is drawn to a cosmetic or pharmaceutical composition comprising a polypeptide or peptide, as set forth herein, and a physiologically acceptable carrier or excipient. Preferably, the compound is present in an amount of about 0.0001 to about 5% by weight based on the total weight of the composition. Also preferably, the compound is present in an amount of about 0.01 to about 0.5% by weight based on the total weight of the composition. The composition may be in the form of an emulsifier or a stabilizing system such as skin softener, a nutrient emulsion, a nutrient cream, a massage cream, treatment serum or a facial pack.

Preferably, the carrier is at least one selected from the group consisting of water, propylene glycol, ethanol, propanol, glycerol, butylene glycol and polyethylene glycol.

In a fourth aspect, the invention is drawn to a method of decreasing VEGF levels in epidermis, the method comprising applying to skin an effective amount of any one of the compounds set forth herein. In a preferred embodiment, the compound or composition is one of the compounds set forth herein.

Brief description of Figures

Figure 1 sets forth a sequence summary of VEGF binding phage clones. Twenty-four phage clones were sequenced after 3 rounds of panning. The sequence alignment tree indicates a highly conserved sequence motif ACXLWPXXWC. The number in parentheses

represents the frequency of that sequence within the 24 clones sequenced after the third round of panning.

Figure 2 sets forth the results of a phage ELISA to demonstrate the binding of unique clones to VEGF and not to BSA. Equivalent amounts of phage were evaluated to determine their relative binding affinity to $hVEGF_{165}$. The clone number and randomized sequence are indicated below each symbol. Target bound phage were detected with anti-M13-HRP. The HRP was monitored with ABTS substrate at an A_{405nm} after 30 minutes (n=3).

Figure 3 sets forth the results of a BIACORE binding analysis of VEGF binding peptides. Binding curves were obtained as described in examples. Data were fit to a two state reaction model with conformation change: Analyte (A) binds to ligand (B) to form complex AB. Complex AB changes to AB* which cannot dissociate directly to A + B. Figure 3A. shows biotinylated peptide CK37281. ka1=$2.84e^3$ $M^{-1}s^{-1}$, kd1=0.0122 s^{-1}, ka2=1.5e-3 s^{-1} kd2=$3.36e^{-3}$ s^{-1} K_D=$1.92e^{-6}$ M. Figure 3 B shows CK37283 (6000 RU VEGF, 3500 RU TNFα no buffer only subtraction); ka1=$1.24e^4$ $M^{-1}s^{-1}$, kd1=0.318 s^{-1}, ka2=6.34e-3 s^{-1}, kd2=$1.23e^{-3}$ s^{-1} K_D=$4.90e^{-6}$ M. Figure 3C shows v114 control peptide (1000 RU VEGF, 850 RU TNFα Data were fit to a 1:1 Langmuir binding ka1=$7.51e^5$ $M^{-1}s^{-1}$ kd1=0.167 s^{-1} K_D=$2.23e^{-7}$ M.

Figure 4 sets forth the plasmid maps of (A) pCB04WT expression phagemid for expression of C-terminal His6X tagged beta-lactamase (B) pME22 N-terminal stuffer phagemid for cloning using Bbs1 restriction sites and (C) pCM01 N-terminal aVEGF-BLA fusion expression phagemid.

Figure 5 sets forth a summary of N-terminal fusion cloning strategy using Bbs1 cloning sites.

Figure 6 shows an SDS-PAGE gel of His-tag purified beta-lactamase fusions with peptides. IMAC purified BLA versions and different peptides were concentrated and loaded onto an SDS PAGE gel (4-12%). Lanes 1 & 10: MW markers. Lane 2: pCB04 (WT with 6Xhis tag). Lanes 3,4,5,6: pCM01 aVEGF-BLA N-terminal fusion protein scaffold. Lanes 7,8: pCM02 achymotrypsin-BLA N-terminal fusion protein.

Figure 7 shows that aVEGF peptide-BLA fusion binds specifically to VEGF. Increasing concentrations of pCM01 (aVEGF peptide-BLA fusion) and pCB04 (WT) were

added to VEGF coated wells of a microtiter plate. Residual bound nitrocefin activity was measured after washing 5X with nitrocefin assay buffer (0.125% n-octyl-beta-D-glucopyranoside in PBS).

Figure 8 shows inhibition of VEGF induced HUVEC proliferation by anti-VEGF peptide (filled circles). Proliferation was monitored by radioactive incorporation of ^{3}H thymidine (n = 3), and anti-VEGF antibody (open circles) was used as a positive control, as seen in the examples.

Figure 9 shows BBI gene sequence designed for efficient cloning. The protein signal sequence is italicized while the trypsin loop (CTKSNPPQC) and chymotrypsin loop (CALSYPAQC) are highlighted in bold.

Figure 10 shows the results of an SDS PAGE of refolded anti-VEGF BBI. Anti-VEGF BBI was refolded in the presence or absence of subtilisin BPN' Y217L. The lanes are as follows: Lane 1: Hampton Foldit 11, refolding buffer, -subtilisn; Lane 2: Hampton Foldit 11 refolding buffer, +subtilsin; Lane 3: Hampton Foldit 13 refolding buffer, -subtilsin; Lane 4, Hampton Foldit 13 refolding buffer, + subtilisn; Lane 5, Molecular Weight Markers.

Figure 11 shows that VEGF-BBI1 (SEQ ID NO: 9) binds specifically to VEGF.

Figure 12 sets forth HUVEC results for designated peptides.

Figure 13 sets forth sequences of three BBI-VEGF fusions, BBI-VEGF1 (SEQ ID NO:9), BBI-VEGF2 (SEQ ID NO:10) and BBI-VEGF12 (SEQ ID NO:11). Fusions BBI-VEGF1 and BBI-VEGF2 have only one of the binding loops replaced; fusion BBI-VEGF12 has both of the binding loops replaced.

Detailed Description of the Preferred Embodiments

The invention will now be described in detail by way of reference only using the following definitions and examples. Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those

described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Practitioners are particularly directed to Sambrook *et al.,* 1989, and Ausubel FM *et al.,* 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols and reagents described, as these may vary.

The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

All publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention.

The term "organism" is used throughout the specification to describe an animal, preferably a human, to whom treatment, including prophylactic treatment, with the compounds according to the present invention is provided. For treatment of those infections, conditions or disease states which are specific for a specific animal such as a human patient, the term organism refers to that specific animal. In most instances, the term organism refers to a human patient.

The term "effective amount" is used throughout the specification to describe concentrations or amounts of compounds according to the present invention which may be used to produce a favorable change in the disease or condition treated, whether that change is a remission, a decrease in growth or size of cancer, tumor or other growth, a favorable physiological result including the clearing up of skin or tissue, or the like, depending upon the disease or condition treated.

The term "angiogenesis" is used throughout the specification to describe the biological processes which result in the development of blood vessels or increase in the vascularity of tissue in an organism. With respect to the present invention, the term angiogenesis is defined as the process through which tumors or other rapidly proliferating tissue derive a blood supply through the generation of microvessels.

The terms "angiogenic disease", "angiogenic disorder" and "angiogenic skin disorder" is used throughout the specification to describe a disorder, generally a skin disorder or related disorder which occurs as a consequence of or which results in increased vascularization in tissue. Oftentimes, the etiology of the angiogenic disease is unkown. However, whether angiogenesis is an actual cause of a disease state or is simply a condition of the disease state is unimportant, but the inhibition of angiogenesis in treating or reversing the disease state or condition is an important aspect of the present invention. Examples of angiogenic skin disorders which may be treated utilizing compounds according to the present invention include, for example, psoriasis, acne, rosacea, warts, eczema, hemangiomas and lymphangiogenesis, among numerous others, including Sturge-Weber syndrome, neurofibromatosis, tuberous sclerosis, chronic inflammatory disease and arthritis. Any skin disorder which has as a primary or secondary characterization, increased vascularization, is considered an angiogenic skin disorder for purposes of the present invention and is amenable to treatment with compounds according to the present invention.

The term "rosacea" is used to describe acne, rosacea or erythematosa characterized by vascular and follicular dilation involving the nose and continguous portions of the cheeks. Rosacea may vary from very mild but persistent erythema to extensive hyperplasia of the sebaceous glands with deep-seated papules and pustules and accompanied by telangiectasia at the affected erythematous sites. Also called hypertrophic rosacea or rhinophyma, depending upon the severity of the condition.

The term "wart" is used to describe a small, usually hard tumerous growth on the skin. Also known as a verrucas, a wart is a flesh-colored growth of the skin which is characterized by circumscribed hypertrophy of the papillae of the corium, with thickening of the malpighian, granulation and keratin layers of the epidermis. Verucca vulgaris, a subset of warts or verruca, is characterized by infection of the keratinocytes with human papillomavirus.

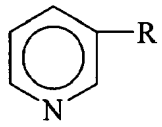
The term "psoriasis" is used to describe a skin condition which is characterized by the eruption of circumscribed, discrete and confluent, reddish, silvery-scaled maculopapules; the lesions occur preeminently on the elbows, knees, scalp and trunk and microscopically show characteristic parakeratosis and elongation of rete ridges.

The term "acne" is used to describe a condition of the skin characterized by inflammatory follicular, papular and pustular eruptions involving the sebaceous apparatus.

Although there are numerous forms of acne, the most common form is known as acne simplex or acne vulgaris which is characeterized by eruptions of the face, upper back and chest and is primarily comprised of comedones, cysts, papules and pustules on an inflammatory base. The condition occurs primarily during puberty and adolesence due to an overactive sebaceous apparatus which is believed to be affected by hormonal activity.

The term "eczema" is a generic term used to describe acute or chronic inflammatory conditions of the skin, typically erythematous, edematous, papular, vesicular and crusting; followed often by lichenification and scaling and occasionally by duskiness of the erythema and, infrequently, hyperpigmentation. Eczema is often accompanied by the sensation of itching and burning. Eczema vesicles form by intraepidermal spongiosis. Eczema is sometimes referred to colloquially as tetter, dry tetter and scaly tetter. There are numerous subcategories of eczema, all of which are treated by one or more of the compounds according to the present invention.

As used herein, "vitamin B_3 compound" means a compound having the formula:



wherein R is - $CONH_2$ (i.e., niacinamide), - COOH (i.e., nicotinic acid) or - CH_2OH (i.e., nicotinyl alcohol); derivatives thereof; and salts of any of the foregoing.

As used herein, "non-vasodilating" means that an ester does not commonly yield a visible flushing response after application to the skin in the subject compositions (the majority of the general population would not experience a visible flushing response, although such compounds may cause vasodilation not visible to the naked eye).

As used herein, "retinoid" includes all natural and/or synthetic analogs of Vitamin A or retinol-like compounds which possess the biological activity of Vitamin A in the skin as well as the geometric isomers and stereoisomers of these compounds.

As used herein, "silicone gum" means high molecular weight silicones having a weight average molecular weight in excess of about 200,000 and preferably from about 200,000 to about 4,000,000. Included are non-volatile polyalkyl and polyaryl siloxane gums.

As used herein, the term "polypeptide" refers to a compound made up of a single chain of amino acid residues linked by peptide bonds. The term "protein" herein may be synonymous with the term "polypeptide" or may refer, in addition, to a complex of two or more polypeptides.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of the gene. The process includes both transcription and translation.

As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, that may or may not include regions preceding or following the coding region.

As used herein, the term "nucleic acid molecule" includes RNA, DNA and cDNA molecules. It will be understood that as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein, such as the mutant proteins of the invention, may be produced.

As used herein, the term "disulfide bridge" or "disulfide bond" refers to the bond formed between the sulphur atoms of cysteine residues in a polypeptide or a protein. In this invention, a disulfide bridge or disulfide bond may be non-naturally occurring and introduced by way of point mutation.

As used herein, the term "salt bridge" refers to the bond formed between oppositely charged residues, amino acids in a polypeptide or protein. In this invention, a salt bridge may be non-naturally occurring and introduced by way of point mutation.

As used herein, an "enzyme" refers to a protein or polypeptide that catalyzes a chemical reaction.

As used herein, the term "activity" refers to a biological activity associated with a particular protein, such as enzymatic activity associated with a protease. Another example might be a protein binding to a receptor which causes measurable downstream effects, such as VEGF binding to its cognate receptor, Flk. Biological activity refers to any activity that would normally be attributed to that protein by one skilled in the art.

As used herein, the term "protease" refers to an enzyme that degrades by hydrolyzing at least some of their peptide bonds.

As used herein, "peptide bond" refers to the chemical bond between the carbonyl group of one amino acid and the amino group of another amino acid.

As used herein, "wild-type" refers to a sequence or a protein that is native or naturally occurring.

As used herein, "point mutations" refers to a change in a single nucleotide of DNA, especially where that change shall result in a sequence change in a protein.

As used herein, "mutant" refers to a version of an organsim or protein where the version is other than wild-type. The change may be affected by methods well known to one skilled in the art, for example, by point mutation in which the resulting protein may be referred to as a mutant.

As used herein, "mutagenesis" refers to the process of affecting a change from a wild-type into a mutant or variant.

As used herein, "substituted" and "modified" are used interchangeably and refer to a sequence, such as an amino acid sequence comprising a polypeptide, that includes a deletion, insertion, replacement or interruption of a naturally occurring sequence. Often in the context of the invention, a substituted sequence shall refer, for example, to the replacement of a naturally occurring residue.

As used herein, "loop" refers to a sequence of amino acids, for example 3-20 amino acids, preferably 5-15 amino acids, preferably 5-10 amino acids, preferably 7-9 amino acids, which connects structural elements of a protein. Such elements may be, for example, beta sheets and helical elements and the connecting loop of a beta-hairpin. The loop may be further stabilized through the use of covalent linkages, specifically by the of disulfide bonds, especially as provided herein. Or, alternatively, for example, the loops may be stabilized by the use of, for example, amides. These loops are typically in the surface of proteins and may be altered, as provided herein, for example, to confer additional properties on the requisite proteins.

As used herein, "CK" followed by an integer shall refer to a specific peptide. Peptide sequences can be found as described herein (*see*, for example, Figure 1). As an example, CK37281 refers to the peptide sequence "ACYNLYGWTCGGG" as shown in Figure 1.

As used herein, "oligonucleotides" refers to a short nucleotide sequence which may be used, for example, as a primer in a reaction used to create mutant proteins.

As used herein, "codon" refers to a sequence of three nucleotides in a DNA or mRNA molecule that represents the instruction for incorporation of a specific amino acid into a polypeptide chain.

In a first aspect, the invention is drawn to a cosmetic or pharmaceutical compound for improving the appearance of skin comprising a polypeptide or a peptide. The polypeptide or peptide may be one that binds to VEGF and blocks its downstream activity. In one embodiment, the compound is a peptide. In another embodiment, the compound is a polypeptide. In a preferred embodiment, the peptide has an amino acid sequence selected from the group consisting of SEQ ID NO:1-SEQ ID NO:7. In a preferred embodiment, the peptide has a conserved binding sequence, the sequence being XXLWPXWC. In a preferred embodiment, the sequence comprises SEQ ID NO:1. In a preferred embodiment, the compound has a sequence, the sequence being at least 70%, preferably 80%, preferably 90%, preferably 95% homologous to the sequences set forth herein. In a preferred embodiment, the polypeptide has a molecular weight that is preferably between 500 Daltons and 30,000 Daltons, more preferably between 1000 Daltons and 10,000 Daltons, most preferably from 1500 Daltons to 8,000 Daltons.

In a preferred embodiment, the compound is used for the improvement of skin in an organism with a skin disorder. In a preferred embodiment, the skin disorder is an angiogenic skin disorder. In a preferred embodiment, the skin disorder is at least one selected from the group consisting of psoriasis, venous ulcers, acne, rosacea, warts, eczema, hemangiomas and lymphangiogenesis, among numerous others. In a preferred embodiment, the skin disorder is rosacea.

In a second aspect, the invention is drawn to a cosmetic or pharmaceutical compound for improving the appearance of skin comprising a peptide or a polypeptide and a scaffold, the peptide or polypeptide being expressed in the scaffold, preferably the peptide or polypeptide being a loop, preferably, the loop being closed by a disulfide bond. The peptide or polypeptide may be one that blocks binds to VEGF and blocks its downstream activity. In a preferred embodiment, the scaffold is STI, Eglin or BBI. In a preferred embodiment, the preferred scaffold is BBI.

In a preferred embodiment, the compound further comprises a peptide. Preferably, the peptide has an amino acid sequence selected from the group consisting of SEQ ID NO: 1- SEQ ID NO: 7. Preferably, the compounds comprises one of SEQ ID NO: 9 through

SEQ ID NO: 11.. Preferably, the compound comprises SEQ ID NO: 9. In a preferred embodiment, the peptide has a conserved binding sequence, the sequence being XXLWPXWC. In a preferred embodiment, the compound has a sequence, the sequence being at least 70%, preferably 80%, preferably 90%, preferably 95% identical to the sequences set forth herein. The peptide molecular weight is preferably between 500 Daltons and 45,000 Daltons, preferably between 1000 Daltons and 12,000 Daltons, preferably from 1500 Daltons to 10,000 Daltons. In a preferred embodiment, the compound preferably comprises a polypeptide.

In a preferred embodiment, the compound is used for the improvement of skin in an organism with a skin disorder. In a preferred embodiment, the skin disorder is an angiogenic skin disorder. In a preferred embodiment, the skin disorder is at least one selected from the group consisting of psoriasis, venous ulcers, acne, rosacea, warts, eczema, hemangiomas, lymphangiogenesis, among numerous others. In a preferred embodiment, the skin disorder is rosacea.

The current invention is directed to a peptide or a polypeptide, a loop and a protease-resistant scaffold. Flexible native loops are found on the surface of most protein modules and exist as short stretches of amino acids that connect regions of defined secondary structure. Although crystallographic and NMR studies show that native loops are usually less well defined than alpha-helices and beta-sheets, their conformational freedom is normally restricted substantially compared with free peptides. Consequently, the binding activities of native loops in proteins usually differ significantly from those of the corresponding linear amino acid sequence .

The loops of the current invention bind proteins, such as VEGF. Binding the loop to the protein prevents the protein from binding to its target. Thus, binding interactions are thought to be disrupted by binding the loop to the protein. As a result, bioactivity can be altered.

The current invention provides scaffolds to stabilize the loops. STI, BBI and EglinC have native loops that bind to and inhibit proteases. STI and BBI native loops may be replaced with the polypeptides and/or peptides of the invention, the loops. As an example, the sequences may be replaced with inhibitors of FGF-5, TGFB-1 and TGFB-2, as well as inhibitors of the complement pathway such as C2, C3, C4 or C5 inhibitors and Compstatin, etc. Additionally, STI and BBI loops native may be replaced with sequences which have

been isolated with techniques such as phage display as, for example, the VEGF-binding proteins disclosed herein.

A native loop may be replaced with a loop which is, for example, 3 to 20 amino acids in length, preferably 5 to 15 amino acids in length, preferably 5 to 10 amino acids in length. Longer sequences may be used as long as they provide binding and/or inhibition. In addition, peptides suitable for use as replacements of the native loop(s) can form constrained loops, i.e., a loop formed by the presence to a disulfide bond between two cysteine residues. In specific embodiments, the peptides are between 7 and 9 amino acids in length. These replacement sequences also provide protease inhibition or binding to the targeted proteins.

There are several advantages to using scaffolds to stabilize peptide sequences. First, biological activity of the peptide may be expected to be higher as a result of limiting peptide flexibility and reducing the entropic cost of fixing the polypeptide sequence in the bioactive conformation. Second, structural information can be obtained by x-ray crystallography to guide affinity maturation. Third, the sequence presented on a structural scaffold may be more resistant to proteolytic degradation in different biological applications. Finally, the chimeric construction can be obtained in large amount in low cost biological expression systems for industrial applications (*see*, for example, Attorney Docket No. GC815P filed November 6, 2003, Express Mail Receipt No. EL 967 262 855 US, and is entitled, "Expression in Filamentous Fungi of Protease Inhibitors and Variants Thereof," which is incorporated by reference herein and to which this application claims priority).

BBI represents a class of protein scaffolds whose binding to proteases is mediated by an exposed native loop that is fixed in a characteristic canonical conformation and which fits into the active site in a manner thought to be similar to that of a substrate (Laskowski & Kato, 1980; Bode & Huber, 1992). The native loop is frequently constrained by the presence of disulfide bridges and/or extensive hydrogen-bonding networks that act to lock the structure into the correct canonical structure. The sequence of this loop determines the specificity of the inhibition, which mirrors the specificity of proteinases for their substrates. For example, most trypsin inhibitors have Arg or Lys as their P1 residue. Inhibitors of the BBI family have a network of conserved disulfide bridges that help form a symmetrical structure of two tricyclic domains (Chen *et al.*, 1992; Werner & Wemmer, 1992; Lin *et al.*, 1993), each containing an independent serine proteinase binding site. The native binding

loop is contained within a region joined by disulfide bridges formed between cysteine residues. The identity of the amino acid residue at the P1 site on each domain is the main determinant of the serine proteinase inhibited. Native domains possess lysine or arginine for trypsin, leucine or tyrosine for chymotrypsin and alanine for elastase (Ikenaka & Norioka, 1986). In addition, serine is highly conserved at the P'1 position and proline at the P'3 position. The individual native loop regions of BBI are well suited for protein loop grafting of previously identified cysteine constrained peptides that bind to targets selectively, as disclosed herein.

Numerous isoforms of BBI have been characterized; SEQ ID NO: 8 (Figure 9) shows the amino acid sequence of BBI backbone used herein comprising approximately 71 amino acid residues. In addition, BBI may be truncated with as many as 10 amino acid residues being removed from either the N- or C- terminal. Any of the isoforms disclosed herein, as well as those known in the art, may be used as a scaffold.

The disclosed invention has several advantages over creation of, for example, chimeric proteins. Transfer of an entire protein can be difficult when, for example, a protein domain of interest carries more than one important biological activity. Maintaining one activity (*e.g.* functionally significant domain-domain interactions) while altering another (*e.g.* high affinity binding to a co-factor or receptor) can be problematic. The invention, as disclosed herein, overcomes that limitation, as loops are transferred, instead of entire domains.

The compounds of the invention may comprise one or more mutations in addition to those set out above. Other mutations, such as deletions, insertions, substitutions, transversions, transitions and inversions, at one or more other locations, may also be included.

The compound may also comprise a conservative substitution that may occur as a like-for-like substitution (e.g., basic for basic, acidic for acidic, polar for polar etc.) Non-conservative substitutions may also occur, i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine, diaminobutyric acid ornithine, norleucine ornithine, pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance.

Deliberate amino acid substitutions may be made on the basis of similarity in amino acid properties (such as polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues) and it is therefore useful to group amino acids together in functional groups. Amino acids can be grouped together based on the properties of their side chain alone. However it is more useful to include mutation data as well. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets can be described in the form of a Venn diagram (Livingstone C.D. and Barton G.J. (1993) "Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation" *Comput.Appl Biosci.* 9: 745-756)(Taylor W.R. (1986) "The classification of amino acid conservation" *J.Theor.Biol.* 119; 205-218). Conservative substitutions may be made, for example according to the table below which describes a generally accepted Venn diagram grouping of amino acids.

Set		Sub-set	
Hydrophobic	F W Y H K M I L V A G C	Aromatic	F W Y H
		Aliphatic	I L V
Polar	W Y H K R E D C S T N Q	Charged	H K R E D
		Positively charged	H K R
		Negatively charged	E D
Small	V C A G S P T N D	Tiny	A G S

Variant amino acid sequences may also include suitable spacer groups inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation involves the presence of one or more amino acid residues in peptoid form.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer

programs can calculate % homology between two or more sequences. % homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux et al 1984 Nuc. Acids Research 12 p387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al., 1999 Short Protocols in Molecular Biology, 4th Ed – Chapter 18), FASTA (Altschul et al., 1990

J. Mol. Biol. 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel et al., 1999, Short Protocols in Molecular Biology, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), Gene 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Embodiments of the first and second aspects of the invention, as disclosed above, provide a nucleic acid encoding any of the compounds, as set forth above, as well as complements thereof. In another preferred embodiment, the invention provides for compositions comprising at least one compound, as disclosed herein, and another ingredient. In another preferred embodiment, the invention provides vectors comprising a compound, as disclosed herein, cells comprising the compound and methods of expressing the compound.

One skilled in the art will be aware of the relationship between nucleic acid sequence and polypeptide sequence, in particular, the genetic code and the degeneracy of this code, and will be able to construct such compounds without difficulty. For example,

one skilled in the art will be aware that for each amino acid substitution in the compound sequence there may be one or more codons which encode the substitute amino acid. Accordingly, it will be evident that, depending on the degeneracy of the genetic code with respect to that particular amino acid residue, one or more compound nucleic acid sequences may be generated corresponding to that compound polypeptide sequence.

Mutations in amino acid sequence and nucleic acid sequence may be made by any of a number of techniques, as known in the art. In particularly preferred embodiments, the mutations are introduced into parent sequences by means of PCR (polymerase chain reaction) using appropriate primers. The parent enzymes may be modified at the amino acid level or the nucleic acid level to generate the compound sequences described herein. Therefore, a preferred embodiment provides for the generation of compounds by introducing one or more corresponding codon changes in the nucleotide sequence encoding a compound.

It will be appreciated that the above codon changes can be made in any compound nucleic acid sequence. For example, sequence changes can be made to any of the homologous sequences described herein.

The compound may comprise the "complete" protein, i.e., in its entire length as it occurs in nature (or as mutated), or it may comprise a truncated form thereof. The compound derived from such may accordingly be so truncated, or be "full-length". The truncation may be at the N-terminal end or the C-terminal end. The compound may lack one or more portions, such as sub-sequences, signal sequences, domains or moieties, whether active or not.

In a yet further alternative, the nucleotide sequence encoding the compound may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al.*, (1981) *Tetrahedron Letters* 22, p 1859-1869 or the method described by Matthes *et al.*, (1984) *EMBO J.* 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin in accordance with standard techniques.

Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al.*, (*Science* (1988) 239, pp 487-491).

The nucleotide sequences described here, and suitable for use in the methods and compositions described here may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of this document, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

A preferred embodiment of the invention provides for nucleotide sequences and the use of nucleotide sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof.

The polynucleotides may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides.

Polynucleotides such as DNA polynucleotides and probes may be produced recombinantly, synthetically or by any means available to those of skill in the art. They may also be cloned by standard techniques. In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA

can be cloned into a suitable cloning vector. Preferably, the variant sequences are at least as biologically active as the sequences presented herein.

A preferred embodiment of the invention includes sequences that are complementary to the compound or sequences that are capable of hybridising either to the nucleotide sequences of the compounds (including complementary sequences of those presented herein), as well as nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of the compounds (including complementary sequences of those presented herein). A preferred embodiment provides polynucleotide sequences that are capable of hybridising to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency.

A preferred embodiment includes nucleotide sequences that can hybridise to the nucleotide sequence of the compound nucleic acid, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC). More preferably, the nucleotide sequences can hybridise to the nucleotide sequence of the compound, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

It may be desirable to mutate the sequence in order to prepare a compound. Accordingly, a mutant may be prepared from the compounds provided herein. Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. A suitable method is disclosed in Morinaga *et al.,* (*Biotechnology* (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (*Analytical Biochemistry* (1989), 180, p 147-151). A further method is described in Sarkar and Sommer (*Biotechniques* (1990), 8, p404-407 – "The megaprimer method of site directed mutagenesis"). Other methods to mutate the sequence are employed and disclosed herein.

In a preferred embodiment, the sequence for use in the methods and compositions described here is a recombinant sequence – i.e. a sequence that has been prepared using recombinant DNA techniques. Such techniques are explained, for example, in the literature, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press.

Another embodiment provides vectors comprising the compound, cells comprising the compound and methods of expressing the compound. The nucleotide sequence for use in the methods and compositions described herein may be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in enzyme form, in and/or from a compatible host cell. Expression may be controlled using control sequences, e.g., regulatory sequences. The enzyme produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences may be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane. Polynucleotides can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. The vector comprising the polynucleotide sequence may be transformed into a suitable host cell. Suitable hosts may include bacterial, yeast, insect and fungal cells.

Compounds and their polynucleotides may be expressed by introducing a polynucleotide into a replicable vector, introducing the vector into a compatible host cell and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell.

The compound nucleic acid may be operatively linked to transcriptional and translational regulatory elements active in a host cell of interest. The compound nucleic acid may also encode a fusion protein comprising signal sequences such as, for example, those derived from the glucoamylase gene from *Schwanniomyces occidentalis,* α-factor mating type gene from *Saccharomyces cerevisiae* and the TAKA-amylase from *Aspergillus oryzae*. Alternatively, the compound nucleic acid may encode a fusion protein comprising a membrane binding domain.

The compound may be expressed at the desired levels in a host organism using an expression vector. An expression vector comprising a compound nucleic acid can be any vector capable of expressing the gene encoding the compound nucleic acid in the selected host organism, and the choice of vector will depend on the host cell into which it is to be introduced. Thus, the vector can be an autonomously replicating vector, i.e. a vector that exists as an episomal entity, the replication of which is independent of chromosomal

replication, such as, for example, a plasmid, a bacteriophage or an episomal element, a minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome.

The expression vector typically includes the components of a cloning vector, such as, for example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences encoding a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression vector may comprise a sequence coding for an amino acid sequence capable of targeting the compound to a host cell organelle such as a peroxisome or to a particular host cell compartment. Such a targeting sequence includes but is not limited to the sequence SKL. For expression under the direction of control sequences, the nucleic acid sequence the compound is operably linked to the control sequences in proper manner with respect to expression.

Preferably, a polynucleotide in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The control sequences may be modified, for example, by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators. The control sequences may in particular comprise promoters.

In the vector, the nucleic acid sequence encoding for the compound is operably combined with a suitable promoter sequence. The promoter can be any DNA sequence having transcription activity in the host organism of choice and can be derived from genes that are homologous or heterologous to the host organism. Examples of suitable promoters for directing the transcription of the modified nucleotide sequence, such as compound nucleic acids, in a bacterial host include the promoter of the *lac* operon of *E. coli,* the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α-amylase gene *(amyL),* the aprE promoter of *Bacillus subtilis,* the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene *(amyM),* the promoters of the *Bacillus amyloliquefaciens* α-amylase gene *(amyQ),* the promoters of the *Bacillus subtilis*

xylA and xylB genes and a promoter derived from a *Lactococcus* sp.-derived promoter including the P170 promoter. When the gene encoding the compound is expressed in a bacterial species such as *E. coli,* a suitable promoter can be selected, for example, from a bacteriophage promoter including a T7 promoter and a phage lambda promoter. For transcription in a fungal species, examples of useful promoters are those derived from the genes encoding the *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger glucoamylase, Rhizomucor miehei lipase, Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase. Examples of suitable promoters for the expression in a yeast species include but are not limited to the Gal 1 and Gal 10 promoters of *Saccharomyces cerevisiae* and the *Pichia pastoris AOX1 or* AOX2 promoters.

Examples of suitable bacterial host organisms are gram positive bacterial species such *as Bacillaceae including Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus lautus, Bacillus megaterium and Bacillus thuringiensis, Streptomyces* species such as *Streptomyces murinus,* lactic acid bacterial species in*cluding Lactococcus* spp. such as *Lactococcus lactis, Lactobacillus* spp. including *Lactobacillus reuteri, Leuconostoc spp., Pediococcus* spp. and *Streptococcus spp.* Alternatively, strains of a gram-negative bacterial species belonging to *Enterobacteria*ceae including *E. coli,* or to *Pseudomonadaceae* can be selected as the host organism.

A suitable yeast host organism can be selected from the biotechnologically relevant yeasts species such as but not limited to yeast species such as *Pichia sp., Hansenula sp or Kluyveromyces, Yarrowinia* species or a species of *Saccharomyces including Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyce* such as, for example, *S. Pombe* species. Preferably a strain of the methylotrophic yeast species *Pichia pastoris* is used as the host organism. Preferably the host organism is a *Hansenula* species. Suitable host organisms among filamentous fungi include species of *Aspergillus, e.g. Aspergillus niger, Aspergillus oryzae, Aspergillus tubigensis, Aspergillus awamori or Aspergillus nidulans.* Alternatively, strains of a *Fusarium* species, e.g. *Fusarium*

oxysporum or of a *Rhizomucor* species such as *Rhizomucor miehei* can be used as the host organism. Other suitable strains include *Thermomyces* and *Mucor* species.

Host cells comprising polynucleotides may be used to express polypeptides, such as the compounds disclosed herein, fragments, homologues, variants or derivatives thereof. Host cells may be cultured under suitable conditions which allow expression of the proteins. Expression of the polypeptides may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG. Polypeptides can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption. Polypeptides may also be produced recombinantly in an *in vitro* cell-free system, such as the TnT™ (Promega) rabbit reticulocyte system (*Roche in vitro systems*).

In a third aspect, the invention is drawn to a cosmetic or pharmaceutical composition comprising a polypeptide or peptide, as set forth herein, and a physiologically acceptable carrier or excipient. Preferably, the compound is present in an amount of about 0.0001 to about 5% by weight based on the total weight of the composition. Also preferably, the compound is present in an amount of about 0.01 to about 0.5% by weight based on the total weight of the composition. The composition may be in the form of an emulsifier or a stabilizing system such as skin softener, a nutrient emulsion, a nutrient cream, a massage cream, treatment serum or a facial pack.

Preferably, the carrier is at least one selected from the group consisting of water, propylene glycol, ethanol, propanol, glycerol, butylene glycol and polyethylene glycol.

Liposomes may comprises, at least, water and one or more ingredients capable of forming lipid bilayer vesicles. Non-limiting examples include: phospholipids, hydrogenated phosphatidylcholine, lecithin, cholesterol and sphingolipids. Preferred liposomes include, without limitation: a) lipoid liposome 0003 (composed of water and lecithin and glycerin); b) lipoid liposome 0300 (composed of water and phosphatidylcholine), c) lipoid liposome 0111 (composed of water, ginkgo balboa leaf extract, denatured alcohol, hydrogenated lecithin and cholesterol) d) anti-irritant liposomes (composed of water, cola acuminata seed extract, bisabolol and phospholipids), e) vitamin C and E liposomes (composed of water, phospholipids, tocopheryl acetate and ascorbyl

palmitate), f) firming liposomes (composed of water, butylene glycol, pyrus malus (Apple) fruit extract, phospholipids, tocopheryl acetate and carbomer) and g) moisturizing liposomes (composed of water, sodium PCA, tocopheryl acetate, xanthan gum, arginine, lysine, glycine and proline).

In a preferred embodiment, the composition further comprises a skin care active. Preferably, such skin care actives include but are not limited to antioxidants, such as tocopheryl and ascorbyl derivatives, biflavinoids, terpenoids, synthetics and the like, vitamins and vitamin derivatives, hydroxyl- and polyhydroxy acids and their derivatives, such as AHAs and BHAs and their reaction products, peptides and polypeptides and their derivatives, such as glycopeptides and lipophilized peptides, heat shock proteins and cytokines, enzymes and enzymes inhibitors and their derivatives, such as proteases, MMP inhibitors, catalases, GluOxy and SOD, amino acids and their derivatives, bacterial, fungal and yeast fermentation products and their derivatives, including mushrooms, algae and seaweed and their derivatives, phytosterols and plant and plant part extracts and their derivatives and phospholipids and their derivatives and delivery systems containing them, as provided herein.

In a preferred embodiment, the skin care active is selected from the group consisting of a Vitamin B3 component, panthenol, Vitamin E, Vitamin E acetate, retinol, retinyl, propionate, retinyl palmitate, retinoic acid, Vitamin C, theobromine, alpha-hydroxyacid, farnesol, phytrantriol, salicylic acid, palmityl peptapeptide-3 and mixtures thereof. In a preferred embodiment, the Vitamin B3 component is niacinamide. The compositions herein may comprise a skin care active at a level from about 0.01% to about 20%, preferably from about 0.01% to about 5% more preferably from about 0.01% to about 2%, by weight.

Exemplary derivatives of the foregoing vitamin B_3 compounds include nicotinic acid esters, including non-vasodilating esters of nicotinic acid, nicotinyl amino acids, nicotinyl alcohol esters of carboxylic acids, nicotinic acid N-oxide and niacinamide N-oxide.

Suitable esters of nicotinic acid include nicotinic acid esters of C_1-C_{22}, preferably C_1-C_{16}, more preferably C_1-C_6 alcohols. The alcohols are suitably straight-chain or branched chain, cyclic or acyclic, saturated or unsaturated (including aromatic), and substituted or unsubstituted. The esters are preferably non-vasodilating.

Non-vasodilating esters of nicotinic acid include tocopherol nicotinate and inositol hexanicotinate; tocopherol nicotinate is preferred. A more complete description of vitamin B_3 compounds is given in WO 98/22085. Preferred vitamin B_3 compounds are niacinamide and tocopherol nicotinate.

Another suitable skin care active is a retinoid. The retinoid is preferably retinol, retinol esters (e.g., C_2 - C_{22} alkyl esters of retinol, including retinyl palmitate, retinyl acetate, retinyl proprionate), retinal, and/or retinoic acid (including all-trans retinoic acid and/or 13-cis-retinoic acid), more preferably retinoids other than retinoic acid. These compounds are well known in the art and are commercially available from a number of sources, e.g., Sigma Chemical Company, and Boehringer Mannheim. Preferred retinoids are retinol, retinyl palmitate, retinyl acetate, retinyl proprionate, retinal, retinoic acid and combinations thereof. More preferred are retinol, retinoic propionate, retinoic acid and retinyl palmitate. The retinoid may be included as the substantially pure material, or as an extract obtained by suitable physical and/or chemical isolation from natural (e.g., plant) sources. When a retinoid is included in the compositions herein, it will typically comprise from about 0.005% to about 2%, more preferably from about 0.01% to about 2% retinoid. Retinol is preferably used in an amount of from about 0.01% to about 0.15%; retinol esters are preferably used in an amount of from about 0.01% to about 2% (e.g., about 1%).

The compositions herein can comprise a safe and effective amount of a dermatologically acceptable carrier, suitable for topical application to the skin or hair within which the essential materials and optional other materials are incorporated to enable the essential materials and optional components to be delivered to the skin or hair at an appropriate concentration. The carrier can thus act as a diluent, dispersant, solvent or the like for the essential components which ensures that they can be applied to and distributed evenly over the selected target at an appropriate concentration.

An effective amount of one or more compounds described herein may also be included in compositions to be applied to keratinous materials such as nails and hair, including but not limited to those useful as hair spray compositions, hair styling compositions, hair shampooing and/or conditioning compositions, compositions applied for the purpose of hair growth regulation and compositions applied to the hair and scalp for the purpose of treating seborrhoea, dermatitis and/or dandruff.

An effective amount of one or more compounds described herein may be included in compositions suitable for topical application to the skin or hair. These compositions can be in the form of creams, lotions, gels, suspensions dispersions, microemulsions, nanodispersions, microspheres, hydrogels, emulsions (e.g., oil in water and water in oil, as well as multiple emusions) and multilaminar gels and the like (*see*, for example, The Chemistry and Manufacture of Cosmetics, Schlossman et al., 1998, incorporated by reference, herein), and may be formulated as aqueous or silicone compositions or may be formulated as emulsions of one or more oil phases in an aqueous continuous phase (or an acqueous phase in an oil phase).

The type of carrier utilized in the present invention depends on the type of product form desired for the composition. The carrier can be solid, semi-solid or liquid. Suitable carriers are liquid or semi-solid, such as creams, lotions, gels, sticks, ointments, pastes, sprays and mousses. Preferably the carrier is in the form of a lotion, cream or a gel, more preferably one which has a sufficient thickness or yield point to prevent the particles from sedimenting. The carrier can itself be inert or it can possess dermatological benefits of its own. The carrier may be applied directly to the skin and/or hair or it may be applied via a woven or non-woven wipe or cloth. It may also be in the form of a patch, mask or wrap. It may also be aerosolized or otherwise sprayed or pumped onto the skin and/or hair. The carrier should also be physically and chemically compatible with the essential components described herein, and should not unduly impair stability, efficacy or other use benefits associated with the compositions of the present invention.

Preferred carriers contain a dermatologically acceptable, hydrophilic diluent. Suitable hydrophilic diluents include water, organic hydrophilic diluents such as $C_2 - C_{10}$, preferably $C_2 - C_6$, preferably, $C_3 - C_6$ monohydric alcohols and low molecular weight glycols and polyols, including propylene glycol, polyethylene glycol polypropylene glycol, glycerol, butylene glycol, 1,2,4-butanetriol, sorbitol esters, 1,2,6-hexametriol, pentylene glycol, hexylene glycol, sorbitol esters, ethoxylated ethers, propoxylated ethers and combinations thereof. The diluent is preferably liquid. Water is a preferred diluent. The composition preferably comprises at least about 20% of the hydrophilic diluent.

Suitable carriers may also comprise an emulsion comprising a hydrophilic phase, especially an aqueous phase, and a hydrophobic phase e.g., a lipid, oil or oily material. As well known to one skilled in the art, the hydrophilic phase will be dispersed in the

hydrophobic phase, or vice versa, to form respectively hydrophilic or hydrophobic dispersed and continuous phases, depending on the composition ingredients. In emulsion technology, the term "dispersed phase" is a term well-known to one skilled in the art which means that the phase exists as small particles or droplets that are suspended in and surrounded by a continuous phase. The dispersed phase is also known as the internal or discontinuous phase. The emulsion may be or comprise (e.g., in a triple or other multi-phase emulsion) an oil-in-water emulsion or a water-in-oil emulsion such as a water-in-silicone emulsion. Oil-in-water emulsions typically comprise from about 1% to about 60% (preferably about 1% to about 30%) of the dispersed hydrophobic phase and from about 1% to about 99% (preferably from about 10% to about 90%) of the continuous hydrophilic phase; water-in-oil emulsions typically comprise from about 1% to about 98% (preferably from about 40% to about 90%) of the dispersed hydrophilic phase and from about 1% to about 50% (preferably about 1% to about 30%) of the continuous hydrophobic phase.

The carrier might also include one or more components that facilitate penetration through the upper stratum corneum barrier to the lower levels of the skin. Examples of penetration enhancers include, but are not limited to, propylene glycol, azone, ethoxydiglycol, dimethyl isosorbide, urea, ethanol and dimetheyl sulfoxide. Other examples include, but are not limited to, micoremulsions, liposomes and nanoemulsions.

The compositions of the present invention may comprise humectants which are preferably present at a level of from about 0.01% to about 20%, preferably from about 0.1% to about 15% and preferably from about 0.5% to about 10%. Preferred humectants include, but are not limited to, compounds selected from polyhydric alcohols, sorbitol, glycerol, urea, D or DL panthenol, calcium pantothenate, royal jelly, panthetine, pantotheine, panthenyl ethyl ether, pangamic acid, pyridoxin, pantoyl lactose Vitamin B complex, sodium pyrrolidone carboxylic acid, hexane - 1, 2, 6, - triol, guanidine or its derivatives, and mixtures thereof.

Suitable polyhydric alcohols for use herein include polyalkylene glycols and preferably alkylene polyols and their derivatives, including propylene glycol, dipropylene glycol, polypropylene glycol, polyethylene glycol and derivatives thereof, sorbitol, hydroxypropyl sorbitol, erythritol, threitol, pentaerythritol, xylitol, glucitol, mannitol, pentylene glycol, hexylene glycol, butylene glycol (e.g., 1,3-butylene glycol), hexane triol (e.g., 1,2,6-hexanetriol), trimethylol propane, neopentyl glycol, glycerine, ethoxylated

glycerine, propane-1,3 diol, propoxylated glycerine and mixtures thereof. The alkoxylated derivatives of any of the above polyhydric alcohols are also suitable for use herein. Preferred polyhydric alcohols of the present invention are selected from glycerine, butylene glycol, propylene glycol, pentylene glycol, hexylene glycol, dipropylene glycol, polyethylene glycol, hexane triol, ethoxylated glycerine and propoxylated glycerine and mixtures thereof.

Suitable humectants useful herein are sodium 2-pyrrolidone-5-carboxylate (NaPCA), guanidine; glycolic acid and glycolate salts (e.g. ammonium and quaternary alkyl ammonium); lactic acid and lactate salts (e.g. ammonium and quaternary alkyl ammonium); aloe vera in any of its variety of forms (e.g., aloe vera gel); hyaluronic acid and derivatives thereof (e.g., salt derivatives such as sodium hyaluronate); lactamide monoethanolamine; acetamide monoethanolamine; urea; panthenol and derivatives thereof; and mixtures thereof.

At least part (up to about 5% by weight of composition) of a humectant can be incorporated in the form of an admixture with a particulate cross-linked hydrophobic acrylate or methacrylate copolymer, itself preferably present in an amount of from about 0.1% to about 10%, which can be added either to the aqueous or disperse phase. This copolymer is particularly valuable for reducing shine and controlling oil while helping to provide effective moisturization benefits and is described in further detail by WO96/03964, incorporated herein by reference.

The oil in water and oil in water emulsion embodiments of the present invention may comprise from about 0.05% to about 20%, preferably from about 1% to about 15%, preferably from about 2% to about 10%, preferably from about 2% to about 5% of a dermatologically acceptable emollient. Emollients tend to lubricate the skin, increase the smoothness and suppleness of the skin, prevent or relieve dryness of the skin and/or protect the skin. Emollients are typically water-immiscible, oily or waxy materials and emollients with high molecular weights can confer aesthetic properties to a topical composition. A wide variety of suitable emollients are known and may be used herein. Sagarin, Cosmetics, Science and Technology, 2nd Edition, Vol. 1, pp. 32-43 (1972), contains numerous examples of materials suitable as an emollient. All emollients discussed in application WO 00/24372 should be considered as suitable for use in the present invention although preferred examples are outlined in further detail below:

i) Straight and branched chain hydrocarbons having from about 7 to about 40 carbon atoms, such as mineral oils, dodecane, squalane, cholesterol, hydrogenated polyisobutylene, isohexadecane, isoeicosane, isooctahexacontane, isohexapentacontahectane, and the C_7-C_{40} isoparaffins, which are C_7-C_{40} branched hydrocarbons. Suitable branched chain hydrocarbons for use herein are selected from isopentacontaoctactane, petrolatum and mixtures thereof.

ii) C_1-C_{30} fatty acid esters of C_1-C_{30} carboxylic acids, C12-15 alkyl benzoates and of C_2-C_{30} dicarboxylic acids, e.g. isononyl isononanoate, isostearyl neopentanoate, isodecyl octanoate, isodecyl isononanoate, tridecyl isononanoate, myristyl octanoate, octyl pelargonate, octyl isononanoate, myristyl myristate, myristyl neopentanoate, myristyl octanoate, isopropyl myristate, myristyl propionate, isopropyl stearate, isopropyl isostearate, methyl isostearate, behenyl behenate, dioctyl maleate, diisopropyl adipate, and diisopropyl dilinoleate and mixtures thereof.

iii) C_1-C_{30} mono- and poly- esters of sugars and related materials. These esters are derived from a sugar or polyol moiety and one or more carboxylic acid moieties. Depending on the constituent acid and sugar, these esters can be in either liquid or solid form at room temperature. Examples include: glucose tetraoleate, the galactose tetraesters of oleic acid, the sorbitol tetraoleate, sucrose tetraoleate, sucrose pentaoleate, sucrose hexaoleate, sucrose heptaoleate, sucrose octaoleate, sorbitol hexaester. Other materials include cottonseed oil or soybean oil fatty acid esters of sucrose. Other examples of such materials are described in WO 96/16636, incorporated by reference herein.

iv) Vegetable oils and hydrogenated vegetable oils. Examples of vegetable oils and hydrogenated vegetable oils include safflower oil, grapeseed oil, coconut oil, cottonseed oil, menhaden oil, palm kernel oil, palm oil, peanut oil, soybean oil, rapeseed oil, linseed oil, rice bran oil, pine oil, nut oil, sesame oil, sunflower seed oil, partially and fully hydrogenated oils from the foregoing sources and mixtures thereof

v) Soluble or colloidally-soluble moisturising agents. Examples include hyalaronic acid, chondroitin and starch-grafted sodium polyacrylates.

Compositions herein may contain an emulsifier and/or surfactant, generally to help disperse and suspend the disperse phase within the continuous aqueous phase. A surfactant

may also be useful if the product is intended for skin cleansing. For convenience hereinafter emulsifiers will be referred to under the term 'surfactants', thus 'surfactant(s)' will be used to refer to surface active agents whether used as emulsifiers or for other surfactant purposes such as skin cleansing. Known or conventional surfactants can be used in the composition, provided that the selected agent is chemically and physically compatible with essential components of the composition and provides the desired characteristics. Suitable surfactants include non-silicone derived materials and mixtures thereof. All surfactants discussed in application WO 00/24372 should be considered as suitable for use in the present invention.

The compositions of the present invention may comprise from about 0.05% to about 15% of a surfactant or mixture of surfactants. The exact surfactant or surfactant mixture chosen will depend upon the pH of the composition, the other components present and the desired final product aesthetics.

Among the nonionic surfactants that are useful herein are those that can be broadly defined as condensation products of long chain alcohols, e.g. C_{8-30} alcohols, with sugar or starch polymers ie glycosides. Other useful nonionic surfactants include the condensation products of alkylene oxides with fatty acids (i.e. alkylene oxide esters of fatty acids). These materials have the general formula $RCO(X)_nOH$ wherein R is a C_{10-30} alkyl group, X is $-OCH_2CH_2-$ (i.e. derived from ethylene glycol or oxide) or $-OCH_2CHCH_3-$ (i.e. derived from propylene glycol or oxide) and n is an integer from about 6 to about 200. Other nonionic surfactants are the condensation products of alkylene oxides with 2 moles of fatty acids (i.e. alkylene oxide diesters of fatty acids). These materials have the general formula $RCO(X)_nOOCR$ wherein R is a C_{10-30} alkyl group, X is $-OCH_2CH_2-$(i.e. derived from ethylene glycol or oxide) or $-OCH_2CHCH_3-$(i.e. derived from propylene glycol or oxide) and n is an integer from about 6 to about 100. An emulsifier for use herein is most preferably a fatty acid ester blend based on a mixture of sorbitan fatty acid ester and sucrose fatty acid ester, especially a blend of sorbiton stearate and sucrose cocoate. Even further suitable examples include a mixture of cetearyl alcohols and cetearyl glucosides.

The hydrophilic surfactants useful herein can alternatively or additionally include any of a wide variety of cationic, anionic, zwitterionic, and amphoteric surfactants such as are known in the art. (See, e.g., McCutcheon's, Detergents and Emulsifiers, North

American Edition (1986), published by Allured Publishing Corporation; U.S. Patent No. 5,011,681 to Ciotti et al., issued April 30, 1991; U.S. Patent No. 4,421,769 to Dixon et al., issued December 20, 1983; and U.S. Patent No. 3,755,560 to Dickert et al., issued August 28, 1973).

A variety of anionic surfactants are also useful herein. (See, e.g., U.S. Patent No. 3,929,678, to Laughlin et al., issued December 30, 1975). Exemplary anionic surfactants include the alkoyl isethionates (e.g., C_{12} - C_{30}), alkyl and alkyl ether sulfates and salts thereof, alkyl and alkyl ether phosphates and salts thereof, alkyl methyl taurates (e.g., C_{12} - C_{30}), and soaps (e.g., substituted alkylamine and alkali metal salts, e.g., sodium or potassium salts) of fatty acids.

Amphoteric and zwitterionic surfactants are also useful herein. Examples of amphoteric and zwitterionic surfactants which can be used in the compositions of the present invention are those which are broadly described as derivatives of aliphatic secondary and tertiary amines in which the aliphatic radical can be straight or branched chain and wherein one of the aliphatic substituents contains from about 8 to about 22 carbon atoms (preferably C_8 - C_{18}) and one contains an anionic water solubilising group, e.g., carboxy, sulfonate, sulfate, phosphate, or phosphonate. Examples are alkyl imino acetates and iminodialkanoates and aminoalkanoates, imidazolinium and ammonium derivatives. Other suitable amphoteric and zwitterionic surfactants are those selected from the group consisting of betaines, sultaines, hydroxysultaines, and branched and unbranched alkanoyl sarcosinates, and mixtures thereof.

Some emulsions of the present invention may include a silicone containing emulsifier or surfactant. A wide variety of silicone emulsifiers are useful herein. These silicone emulsifiers are typically organically modified organopolysiloxanes, also known to those skilled in the art as silicone surfactants. Useful silicone emulsifiers include dimethicone copolyols. These materials are polydimethyl siloxanes which have been modified to include polyether side chains such as polyethylene oxide chains, polypropylene oxide chains, mixtures of these chains and polyether chains containing moieties derived from both ethylene oxide and propylene oxide. Other examples include alkyl-modified dimethicone copolyols, i.e., compounds which contain C_2-C_{30} pendant side chains. Still

other useful dimethicone copolyols include materials having various cationic, anionic, amphoteric, and zwitterionic pendant moieties.

The compositions of the present invention can comprise at least one polymeric thickening agent. The polymeric thickening agents useful herein preferably have a number average molecular weight of greater than about 20,000, preferably greater than about 50,000 and preferably greater than about 100,000. The compositions of the present invention may comprise from about 0.01% to about 10%, preferably from about 0.1% to about 8% and preferably from about 0.5% to about 5% by weight of the composition of the polymeric thickening agent or mixtures thereof.

Preferred polymer thickening agents for use herein include non-ionic thickening agents and anionic thickening agents or mixtures thereof. Suitable non-ionic thickening agents include polyacrylamide polymers, crosslinked poly(N-vinylpyrrolidones), polysaccharides, natural or synthetic gums, polyvinylpyrrolidone and polyvinylalcohol. Suitable anionic thickening agents include acrylic acid/ethyl acrylate copolymers, carboxyvinyl polymers and crosslinked copolymers of alkyl vinyl ethers and maleic anhydride. As an example, Noveon sells a thickener under the trade mark of Carbopol resins or mixtures thereof. Suitable Carbopol resins may be hydrophobically modified, and other suitable resins are described in WO98/22085, or mixtures thereof.

The present compositions may comprise at least one silicone oil phase. Silicone oil phase(s) generally comprises from about 0.1% to about 20%, preferably from about 0.5% to about 10%, preferably from about 0.5% to about 5%, of the composition. The silicone oil phase preferably comprises one or more silicone components.

Silicone components can be fluids, including straight chain, branched and cyclic silicones. Suitable silicone fluids useful herein include silicones inclusive of polyalkyl siloxane fluids, polyaryl siloxane fluids, cyclic and linear polyalkylsiloxanes, poly-alkoxylated silicones, amino and quaternary ammonium modified silicones, polyalkylaryl siloxanes or a polyether siloxane copolymer and mixtures thereof. The silicone fluids can be volatile or non-volatile. Silicone fluids generally have an average molecular weight of less than about 200,000. Suitable silicone fluids have a molecular weight of about 100,000 or less, preferably about 50,000 or less, preferably about 10,000 or less. Preferably the silicone fluid is selected from silicone fluids having a weight average molecular weight in the range from about 100 to about 50,000 and preferably from about 200 to about 40,000.

Typically, silicone fluids have a viscosity ranging from about 0.65 to about 600,000 mm^2s^{-1}, preferably from about 0.65 to about 10,000 $mm^2.s^{-1}$ at 25°C. The viscosity can be measured by means of a glass capillary viscometer as set forth in Dow Corning Corporate Test Method CTM0004, July 29, 1970. Suitable polydimethyl siloxanes that can be used herein include those available, for example, from the General Electric Company and from Dow Corning. Also useful are essentially non-volatile polyalkylarylsiloxanes, for example, polymethylphenylsiloxanes, having viscosities of about 0.65 to 30,000 mm^2s^{-1} at 25°C. These siloxanes are available, for example, from the General Electric Company or from Dow Corning. Cyclic polydimethylsiloxanes suitable for use herein are those having a ring structure incorporating from about 3 to about 7 $(CH_3)_2SiO$ moieties, preferably about 5 or more.

Silicone gums can also be used herein. In preferred embodiments, a silicone oil phase comprises a silicone gum or a mixture of silicones including the silicone gum. Typically, silicone gums have a viscosity at 25°C in excess of about 1,000,000 mm^2s^{-1}. The silicone gums include dimethicones as described by Petrarch and others including US-A-4,152,416, May 1, 1979 to Spitzer, et al, and Noll, Walter, Chemistry and Technology of Silicones, New York: Academic Press 1968. Also describing silicone gums are General Electric Silicone Rubber Product Data Sheets SE 30, SE 33, SE 54 and SE 76. Specific examples of silicone gums include polydimethylsiloxane, (polydimethylsiloxane)-(methylvinylsiloxane) copolymer, poly(dimethylsiloxane)(diphenyl)(methylvinylsiloxane) copolymer and mixtures thereof. Preferred silicone gums for use herein are silicone gums having a molecular weight of from about 200,000 to about 4,000,000 selected from dimethiconol, dimethicone copolyol, dimethicone and mixtures thereof.

A silicone phase herein preferably comprises a silicone gum incorporated into the composition as part of a silicone gum-fluid blend. When the silicone gum is incorporated as part of a silicone gum-fluid blend, the silicone gum preferably constitutes from about 5% to about 40%, especially from about 10% to 20% by weight of the silicone gum-fluid blend. Suitable silicone gum-fluid blends herein are mixtures consisting essentially of:

(i) a silicone having a molecular weight of from about 200,000 to about 4,000,000 selected from dimethiconol, fluorosilicone and dimethicone and mixtures thereof; and

(ii) a carrier which is a silicone fluid, the carrier having a viscosity from about 0.65 mm^2s^{-1} to about 100 mm^2s^{-1},

wherein the ratio of i) to ii) is from about 10:90 to about 20:80 and wherein said silicone gum-based component has a final viscosity of from about 100 mm^2s^{-1} to about 100,000 mm^2s^{-1}, preferably from 500 mm^2s^{-1} to about 10,000 mm^2s^{-1}.

Further silicone components suitable for use in a silicone oil phase herein are crosslinked polyorganosiloxane polymers, optionally dispersed in a fluid carrier. In general, when present the crosslinked polyorganosiloxane polymers, together with its carrier (if present) comprises from about 0.1% to about 20%, preferably from about 0.5% to about 10%, preferably from about 0.5% to about 5% of the composition. Such polymers comprise polyorganosiloxane polymers crosslinked by a crosslinking agent. Suitable crosslinking agents are disclosed in WO98/22085. Examples of suitable polyorganosiloxane polymers for use herein include methyl vinyl dimethicone, methyl vinyl diphenyl dimethicone and methyl vinyl phenyl methyl diphenyl dimethicone.

Another class of silicone components suitable for use in a silicone oil phase herein includes polydiorganosiloxane-polyoxyalkylene copolymers containing at least one polydiorganosiloxane segment and at least one polyoxyalkylene segment. Suitable polydiorganosiloxane segments and copolymers thereof are disclosed in WO98/22085. Suitable polydiorganosiloxane-polyalkylene copolymers are available commercially under the tradenames Belsil (RTM) from Wacker-Chemie GmbH. A particularly preferred copolymer fluid blend for use herein includes Dow Corning DC3225C which has the CTFA designation Dimethicone/Dimethicone copolyol.

Compositions of the present invention may comprise an organic sunscreen. Suitable sunscreens can have UVA absorbing properties, UVB absorbing properties or a mixture thereof. The exact amount of the sunscreen active will vary depending upon the desired Sun Protection Factor, ie the "SPF" of the composition as well as the desired level of UV protection. SPF is a commonly used measure of photoprotection of a sunscreen against erythema. The SPF is defined as a ratio of the ultraviolet energy required to produce minimal erythema on protected skin to that required to produce the same minimal erythema on unprotected skin in the same individual. Amounts of the sunscreen used are typically from about 2% to about 20%, more typically from about 4% to about 14%. Suitable

sunscreens include, but are not limited to those approved for use in the United States, Japan, Europe and Australia. The compositions of the present invention preferably comprise an SPF of about 2 to about 30, preferably about 4 about 30, preferably about 4 to about 15.

The compositions of the present invention may include one or more UVA absorbing sunscreen actives which absorb UV radiation having a wavelength of from about 320nm to about 400nm. Suitable UVA absorbing sunscreen actives are selected from dibenzoylmethane derivatives, anthranilate derivatives such as methylanthranilate and homomethyl, 1-N-acetylanthranilate, and mixtures thereof. Examples of dibenzoylmethane sunscreen actives are described in Sunscreens: Development, Evaluation, and Regulatory Aspects edited by N. J. Lowe and N. A. Shaath, Marcel Dekker, Inc. The UVA absorbing sunscreen active is preferably present in an amount to provide broad spectrum UVA protection either independently, or in combination with, other UV protective actives which may be present in the composition.

Suitable UVA sunscreen actives are dibenzoylmethane sunscreen actives and their derivatives. They include, but are not limited to, those selected from 2-methyldibenzoylmethane, 4-methyldibenzoylmethane, 4-isopropyldibenzoylmethane, 4-tert-butyldibenzoylmethane, 2, 4-dimethyldibenzoylmethane, 2, 5-dimethyldibenzoylmethane, 4, 4'-diisopropylbenzoylmethane, 4-(1, 1-dimethylethyl)-4'-methoxydibenzoylmethane, 2-methyl-5-isopropyl-4'-methoxydibenzoylmethane, 2-methyl-5-tert-butyl-4'-methoxy-dibenzoylmethane, 2, 4-dimethyl-4'-methoxydibenzoylmethane, 2, 6-dimethyl-4'-tert-butyl-4'methoxydibenzoylmethane, and mixtures thereof. Preferred dibenzoyl sunscreen actives include those selected from 4-(1, 1-dimethylethyl)-4'-methoxydibenzoylmethane, 4-isopropyldibenzoylmethane, and mixtures thereof. A preferred sunscreen active is 4-(1, 1-dimethylethyl)-4'-methoxydibenzoylmethane.

The sunscreen active 4-(1, 1-dimethylethyl)-4'-methoxydibenzoylmethane, which is also known as butyl methoxydibenzoylmethane or Avobenzone, is commercially available under the names of Parsol® 1789 from Givaudan Roure (International) S. A. and Eusolex® 9020 from Merck & Co., Inc. The sunscreen 4-isoproplydibenzoylmethane, which is also known as isopropyldibenzoylmethane, is commercially available from Merck under the name of Eusolex® 8020.

The compositions of the present invention may further include one or more UVB sunscreen actives which absorbs UV radiation having a wavelength of from about 290nm to abut 320nm. The compositions comprise an amount of the UVB sunscreen active which is safe and effective to provide UVB protection either independently, or in combination with, other UV protective actives which may be present in the compositions. The compositions may comprise from about 0.1% to about 16%, preferably from about 0.1% to about 12%, and preferably from about 0.5% to about 8% by weight, of each UVB absorbing organic sunscreen.

A variety of UVB sunscreen actives are suitable for use herein. Nonlimiting examples of such organic sunscreen actives are described in US Patent No 5,087,372 issued February 11, 1992 to Haffey et al.; and US Patent Nos 5,073,371 and 5,073,372 both issued on December 17, 1991 to Turner et al. Still other useful sunscreens are those disclosed in U.S. Patent No. 4,937,370, to Sabatelli, issued June 26, 1990; and U.S. Patent No. 4,999,186, to Sabatelli et al., issued March 12, 1991. Preferred UVB sunscreen actives are selected from 2-ethylhexyl-2-cyano-3, 2-ethylhexyl N,N-dimethyl-p-aminobenzoate, p-aminobenzoic acid, oxybenzone, homomenthyl salicylate, octyl salicylate, 4,4'-methoxy-t-butyldibenzoylmethane, 4-isopropyl dibenzoylmethane, 3-benzylidene camphor, 3-(4-methylbenzylidene) camphor, 3 -diphenylacrylate, 2-phenyl-benzimidazole-5-sulphonic acid (PBSA), cinnamates and their derivatives such as 2-ethylhexyl-p-methoxycinnamate and octyl-p-methoxycinnamate, TEA salicylate, ethylhexyl salycilate, octyldimethyl PABA, camphor derivatives and their derivatives, and mixtures thereof. Preferred organic sunscreen actives are 2-ethylhexyl-2-cyano-3, 3-diphenylacrylate, 2-phenyl- benzimidazole-5-sulphonic acid (PBSA), octyl-p-methoxycinnamate, and mixtures thereof. Salt and acid neutralised forms of the acidic sunscreens are also useful herein.

An agent may also be added to any of the compositions useful in the present invention to stabilise the UVA sunscreen to prevent it from photo-degrading on exposure to UV radiation and thereby maintaining its UVA protection efficacy. A wide range of compounds have been cited as providing these stabilising properties and should be chosen to compliment both the UVA sunscreen and the composition as a whole. Suitable stabilising agents include, but are not limited to, those described in US Patents Nos 5,972,316; 5,968,485; 5,935,556; 5,827,508 and Patent WO 00/06110. Preferred examples of stabilising agents for use in the present invention include 2-ethylhexyl-2-cyano-3, 3-

diphenylacrylate, ethyl-2-cyano-3, 3-diphenylacrylate, 2-ethylhexyl-3, 3-diphenylacrylate, ethyl-3, 3-bis(4-methoxyphenyl)acrylate, diethylhexyl 2,6 napthalate and mixtures thereof (Symrise Chemical Company).

An agent may also be added to any of the compositions useful in the present invention to improve the skin substantivity of those compositions, particularly to enhance their resistance to being washed off by water or rubbed off. Examples include, but are not limted to, acrylates/C12-22 alkylmethacrylate copolymer, acrylate/acrylate copolymer, dimethicone, dimethiconol, Grft-copoly (dimethylsiloxane/I-butyl methacrylate), laurel dimethicone, PVP/Headecane copolymer, PVP/Eicosene copolymer, tricontanyl PVP and trimethoxysiloxysilacate.

In addition to the organic sunscreens compositions of the present invention can additionally comprise inorganic physical sunblocks. Nonlimiting examples of suitable physical sunblocks are described in CTFA International Cosmetic Ingredient Dictionary, 6th Edition, 1995, pp. 1026-28 and 1103, Sayre, R. M. et al., "Physical Sunscreens", J. Soc. Cosmet. Chem., vol 41, no 2, pp. 103-109 (1990) and Lowe et al., as per above. Preferred inorganic physical sunblocks are zinc oxide and titanium dioxide and mixtures thereof.

When used, the physical sunblocks are present in an amount such that the present compositions are transparent on the skin (ie non-whitening), preferably from about 0.5% to about 20%, preferably from about 1% to about 10%. When titanium dioxide is used, it can have an anatase, rutile or amorphous structure. Manufacturers of micornized grade titanium dioxide (zinc oxide) for sunscreen use include, but are not limited to Tayca Corporation, Uniqema, Shinetsu Chemical Corporation, Kerr-McGee, Nanophase, Nanosource, Sachtleben and BASF Corporation, as well as their distribution agents and those companies that further process the material for sunscreen use. Physical sunblock particles, e.g., titanium dioxide and zinc oxide, can be uncoated or coated with a variety of materials including but not limited to amino acids, aluminium compounds such as alumina, aluminium stearate, aluminium laurate, and the like; carboxylic acids and their salts e.g., stearic acid and its salts; phospholipids, such as lecithin; organic silicone compounds; inorganic silicone compounds such as silica and silicates and mixtures thereof. The compositions of the present invention may comprise from about 0.1% to about 15%, more preferably from about 0.1% to about 7% and preferably from about 0.5% to about 5%, by weight, of inorganic sunscreen.

The composition of the present invention may also include preservatives. Such preservatives include, but are not limited to pentylene glycol, ethylene diamine tetra acetate (EDTA) and their salts, chlorhexidine (diacetate, dihydrochloride, digluconate), 1,1,1-trichloro-2-methyl-2-propanol, parachloro metaxylenol, polyhexamethylenebiguanide hydrochloride, dehydroacetic acid, diazolidinyl urea, 2,4-dichlorobenzyl alcohol, 4,4-dimethyl-1,3-oxazolidine, formaldehyse, 37% aqueous solution, 10-15% MeOH to avoid polymerization, glutaradehyde, dimethylidantoin, imidazolidinyl urea, 5-Chloro-2-methyl-4-isothiazolin-3-one, ortho-phenylphenol, 4-hydroxybenzoic acid esters (methyl, ethyl, propyl,isopropyl, butyl, isobutyl) ans salts, trichlosan, 2-phenoxyethanol, phenyl mercuric acetate, borate, nitrate, quaternium 15, salycilate, salicylic acid, calcium, sorbic acid, calcium sorbate, zinc pyrithione, phenoxyethanol and chloroxylenol, diazolidinyl urea, methylparaben, propylparaben and PG, isopropylparabens, isobutylparabens and butylparabens, methylparaben, ethylparaben, propylparaben and butylparaben, phenoxyethanol and methylparaben, ethylparaben, propylparaben, butyl paraben.

A variety of optional ingredients such as neutralising agents, perfumes, and colouring agents, can also be added to the compositions herein. It is preferred that any additional ingredients enhance the skin softness / smoothness benefits of the product. In addition it is preferred that any such ingredients do not negatively impact the aesthetic properties of the product.

Neutralizing agents suitable for use in neutralizing acidic group containing hydrophilic gelling agents herein include sodium hydroxide, potassium hydroxide, ammonium hydroxide, monoethanolamine, diethanolamine, amino methyl propanol, tris-buffer and triethanolamine.

Other optional materials include keratolytic agents; water-soluble or solubilizable preservatives preferably at a level of from about 0.1% to about 5%, such as Germall 115, methyl, ethyl, propyl and butyl esters of hydroxybenzoic acid, benzyl alcohol, DMDM hydantoin iodopropanyl butylcarbanate available under the trade name Glydant Plus from Lonza, EDTA, Euxyl (RTM) K400, Bromopol (2-bromo-2-nitropropane-1,3-diol) and phenoxypropanol; anti-bacterials such as Irgasan (RTM) and phenoxyethanol (preferably at levels of from about 0.1% to about 5%); soluble or colloidally-soluble moisturising agents such as hylaronic acid and starch-grafted sodium polyacrylates such as Sanwet (RTM) IM-1000, IM-1500 and IM-2500 available from Celanese Superabsorbent Materials, Portsmith,

VA, USA and described in USA-A-4,076,663; vitamins such as vitamin A, vitamin C, vitamin E and derivatives thereof and building blocks thereof such as phytantriol and vitamin K and components thereof such as the fatty alcohol dodecatrienol; alpha and beta hydroxyacids; aloe vera; sphingosines and phytosphingosines, cholesterol; skin whitening agents; N-acetyl cysteine; colouring agents; antibacterial agents such as TCC/TCS, also known as triclosan and trichlorocarbon; perfumes and perfume solubilizers. Examples of alpha hydroxy acids include glycolic acid, lactic acid, malic acid, citric acid, glycolic acid in conjunction with ammonium glycolate, alpha-hydroxy ethanoic acid, alpha-hydroxyoctanoic acid, alpha-hydroxycaprylic acid, hydroxycaprylic acid, mixed fruit acid, tri-alp0ha hydroxy fruit acids, triple fruit acid, sugar cane extract, alpha hydroxy and botanical comprise, l-alpha hydroxy acid and glycomer in crosslinked fatty acids alpha nutrium. Preferred examples of alpha hydroxy acids are glycolic acid and lactic acid. It is preferred that alpha hydroxy acids are used in levels of up to about 10%.

Other optional materials include water-soluble or solubilizable preservatives preferably at a level of from about 0.1% to about 5% each, such as Germall 115, methyl, ethyl, propyl and butyl esters of hydroxybenzoic acid, benzyl alcohol, DMDM hydantoin iodopropanyl butylcarbanate available under the trade name Glydant Plus from Lonza, EDTA, Euxyl (RTM) K400, Bromopol (2-bromo-2-nitropropane-1,3-diol), pentylene glycol and phenoxypropanol; anti-bacterials such as Irgasan (RTM) and phenoxyethanol (preferably at levels of from 0.1% to about 5%). Antibacterial agents such as TCC/TCS, also known as triclosan and trichlorocarbon are also useful in compositions of the present invention.

Optional materials include pigments which, where water-insoluble, contribute to and are included in the total level of oil phase ingredients. Pigments suitable for use in the compositions of the present invention can be organic and/or inorganic. Also included within the term pigment are materials having a low color or luster, such as matter finishing agents and also light scattering agents. Further examples of suitable pigments are titanium dioxide, predispersed titanium dioxide, iron oxides, acylglutamate iron oxides, ultramarine blue, D&C dyes, carmine, and mixtures thereof. Depending upon the type of composition, a mixture of pigments will normally be used. The preferred pigments for use herein from the viewpoint of moisturisation, skin feel, skin appearance and emulsion compatibility are

treated pigments. The pigments can be treated with compounds, including but not limited to amino acids, silicones, lecithin and ester oils.

Suitably, the pH of the compositions herein is in the range from about 3.5 to about 10, preferably from about 4 to about 8, preferably from about 5 to about 7, wherein the pH of the final composition is adjusted by addition of acidic, basic or buffer salts as necessary, depending upon the composition of the forms and the pH-requirements of the compounds.

The compositions of the present invention are prepared by standard techniques well known to those skilled in the art. In general the aqueous phase and/ or the oil phase would be prepared separately, with materials of similar phase partitioning being added in any order. If the final product is an emulsion, the two phases will then be combined with vigorous stirring. Any ingredients in the formulation with high volatility, or which are susceptible to hydrolysis or decomposition at high temperatures, can be added with gentle stirring towards the end of the process, post emulsification if applicable.

Dosage frequency and amount will depend upon the desired performance criteria.

In a fourth aspect, the invention is drawn to a method of decreasing VEGF levels in epidermis, the method comprising applying to skin an effective amount of any one of the compounds or compositions disclosed herein.

EXAMPLES

Dermatological Compositions may be prepared as provided below.

MOISTURISING BODYWASH **pH = 7**

RAW MATERIAL	Amount
Deionised Water	QS
Glycerin	4.0
PEG-6 Caprylic/Capric Glycerides	4.0
Palm Kernal Fatty acids	3.0
Sodium Laureth-3 Sulphate	45.0
Cocamide MEA	3.0
Sodium Lauroamphoacetate	25.0
Soyabean Oil	10.0
Polyquaternium-10 (JR30M)	0.70
Compound	1000ppm

BODYWASH

RAW MATERIAL	pH 6.5 Amount	pH 7 Amount	pH 8.5 Amount
Deionised water	QS	QS	QS
Sodium Laureth Sulphate	12	15	8
Cocamidopropyl Betaine	8	10	15
APG Glucoside (Plantacare 2000 1)	0	2	1
Polyquaternium-10 (JR30M)	0.25	0	0
Polyquaternium-7 (Mackam 55)	0	0	0.7
Compound	250ppm	500ppm	1000ppm

BODY LOTION	pH 7	pH 7	pH 7.5	pH 7
RAW MATERIAL	**Amount**	**Amount**	**Amount**	**Amount**
DEIONISED WATER	QS	QS	QS	QS
GLYCERINE	8	8	10	12
ISOHEXADECANE	3	3	3	6
NIACINAMIDE	0	3	5	6
ISOPROPYL ISOSTEARATE	3	3	3	3
Polyacrylamide, Isoparaffin, Laureth-7 (Sepigel 305 [2])	3	3	3	3
PETROLATUM	4	4	4	2
NYLON 12	2	2	2.5	2.5
DIMETHICONE (DC1403 [4])	2	2	2.5	2.5
SUCROSE POLYCOTTONSEED OIL	1.5	1.5	1.5	1.5
Stearyl Alcohol 97%	1	1	1	1
D PANTHENOL	1	1	1	1
DL-alphaTOCOPHEROL ACETATE	1	1	1	1
Cetyl Alcohol 95%	0.5	0.5	0.5	1
BEHYNYL ALCOHOL	1	1	1	0.5
EMULGADE PL 68/50	0.4	0.4	0.5	0.5
STEARIC ACID	0.15	0.15	0.15	0.15
Peg-100-stearate (MYRJ 59 [1])	0.15	0.15	0.15	0.15
Compound				

ULTRA-HIGH MOISTURISING FACIAL CREAM/LOTION **pH 7** **pH 7**

RAW MATERIAL	Amount	Amount
Deionised water	QS	QS
Glycerin	12	5
PEG 400 [6]	0	10
Niacinamide	5	7
Isohexadecane	5	5
Dimethicone (DC1403 [3])	3	2
Polyacrylamide, Isoparaffin, Laureth-7 (Sepigel 305 [1])	3	3
Isopropyl Isostearate	2	2
Polymethylsilsesquioxane	2	2
Cetyl Alcohol 95%	1	1
Sucrose polycottonseed oil	1	1
D-Panthenol	1	1
Vitamin E (Tocopherol Acetate)	1	1
Stearyl Alcohol 95%	0.5	0.5
Cetearyl Glucoside	0.5	0.5
Titanium dioxide	0.3	0.3
Stearic Acid	0.15	0.15
PEG-100-Stearate (Myrj 59 [4])	0.15	0.15

Compound

MOISTURISING CREAM	pH 7	pH 7	pH 7.5
RAW MATERIAL	Amount	Amount	Amount
Deionised water	QS	QS	QS
Glycerin	3	5	10
Petrolatum	3	3	0
Cetyl Alcohol 95%	1.5	1.5	1
Dimethicone Copolyol (DC 3225C[4])	2	2	2
Isopropyl Palmitate	1	1	0.5
Carbomer 954 2	0.7	0.7	0.7
Dimethicone (DC 200/350cs[4])	1	1	1
Stearyl Alcohol 97%	0.5	0.5	1
Stearic acid	0.1	0.1	0.1
Peg-100-stearate (MYRJ 59 [1])	0.1	0.1	0.1
Titanium Dioxide	0.3	0.3	0.3
Compound	50ppm	250ppm	1000ppm

Panning of a phage displayed peptide library

A commercially available phage peptide library PhD C7C (New England Biolabs, Beverly, MA) was panned against $hVEGF_{165}$ (R&D systems) for 3 rounds according to the manufacturers instructions. This procedure yielded the sequence profiles summarized in Figure 1. Individual clones were confirmed using phage ELISA according to the manufacturers instructions, Figure 2

BIAcore™ binding analysis: anti-VEGF peptides

Affinities of the peptides for VEGF were measured using a BIAcore™-3000 surface plasmon resonance system (Biacore, Inc., Piscataway, NJ; *see* Figure 3). A CM5 sensor chip was conditioned with 50 mM NaOH and activated for covalent coupling of VEGF

using *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions (Biacore, Inc., Piscataway, NJ). Human $VEGF_{165}$, (Biosource, Int., Camarillo, CA) was diluted to 5 µg/mL in 20 mM sodium acetate, pH 4.8 and injected at a flow rate of 2 µL/min to achieve approximately 1000 to 6000 response units (RU) of coupled protein. TNFα (human TNFα, Biosource, Int., Camarillo, CA) was similarly coupled to the CM5 sensor chip to approximately 850 to 3500 RU in the reference lane. A solution of 1 M ethanolamine was injected as a blocking agent.

Peptides were synthesized using standard FMOC chemistry, purified by reverse phase HPLC to >95% purity (SynPep, Dublin, CA), and stored at 10 mg/mL in DMSO. For kinetic measurements, twofold serial diluted peptides in HBS-EP buffer, 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (Biacore, Inc., Piscataway, NJ), were injected at 25°C at a flow rate of 20 µL/min. Two-fold serial diluted DMSO samples and buffer samples were also injected for background subtraction. Kinetic parameters were calculated using BIAevaluation 3.1 software.

Construction of a peptide-BLA scaffolds

Plasmid pCM01 (5.1kb) encodes a 15-amino acid peptide sequence CK37281 fused to the N-terminus of *Enterobacter cloaceae* ß-lactamase (BLA) with a pIII signal sequence and C-terminal 6XHis tag, (Figure 4). The plasmid also carries a chloramphenicol resistance gene (CAT) as a selectable marker and expression is driven by a lac promoter (Plac). Plasmid pCM01 was constructed using a Bbs1 vector, pME30 constructed from pCB04. pCB04 was digested with *Dra*III and *Spe* I (New England Biolabs, Beverly, MA) resulting in 2.8 kb and 2.1kb frag fragments. To make the inserts the oligo pairs NtermStf2-F and NtermStf2-R (5 uM) were combined in 50 ul total volume in water, the mixture was heated at 95C in heat block for 5 minutes, and the block was allowed to cool to room temp.

Oligos: NtermStf2-F and NtermStf2-R for stuffer vector insert

NtermStf2-F

5'[Phos]

CTAGTGTCTTCGATCAAGTCGACAACAGCCTGTCTGCAGATCCTGAAGACTGGC

GGAGGTGGTCGCGAATACGATTACCCCGCTGATGAAAGCACAGA **3'**

NtermStf2-R

5'[Phos]
GTGCTTTCATCAGCGGGGTAATCGTATTCGCGACCACCTCCGCCAGTCTTCAGG
ATCTGCAGACAGGCTGTTGTCGACTTGATCGAAGACA **3'**

The 2.8kb frag, 2.1kb frag, and stuffer insert (100bp) were ligated overnight at 16 C in a 1:1:5 molar ratio respectively using 10 µl of the DNA mix and 10 ul of Takara solution I ligase. Ligations were purified using Zymo Research DNA clean kit and eluted in 2x 8ul of water. 5 µl of ligation mix was transformed into 50 µl Top 10 electrocompetent cells (Invitrogen), 250 µl SOC was added and the cells grown forl hr at 37 °C. The transformation mix was diluted 1/10 and plated on both LA + 5 ppm CMP and LA + 5 ppm CMP + 0.1 ppm CTX plates, followed by incubation overnight at 37 °C. 12 colonies were picked from CMP plates, grown in LB + 5 ppm CMP, DNA was isolated and digested with BbsI enzyme (2 sites in stuffer plasmid). pCB04 (WT) was also digested as control. One clone had the correct sequence and was designated pME22.

The VEGF peptide-BLA expression plasmid pCM01 was constructed from pME22 using the following primers for the BBs1 insert (Figure 5):
Oligos VegF-F, VegF-5R, VegF-3RP for peptide insert

VegF-F

5'ACTAGTCGTTCCTTTCTATTCTCACTCTGCTTGTACCCTGTGGCCGACCTTCTG
GTGCGGTGGAGGTTCGACGCCAGTGTCAGAAAAACAGCTG **3'**

VegF-5R

5' AGCAGAGTGAGAATAGAAAGGAACGAC **3'**

VegF-3RP

5' [Phos]CCGCCAGCTGTTTTTCTGACACTGG **3'**

BLA-peptide fusion proteins pCM01 and pCB04 (WT) and a biased library pCM04 were expressed in *E. coli* (TOP10; Invitrogen) in 1-L shake flasks in the presence of 5 ppm CMP and 0.1 ppm cefotoxime antibiotic at 25 °C for 40 hrs. Cell pastes were harvested from the 200 ml cell cultures by centrifugation at 3,000*g* for 10 min. The pastes were then treated with 25 ml of B-PER reagent (Pierce) for 40 min with slow mixing. The extract was separated by centrifugation at 20,000*g* for 20 min. BLA activity of all liquid fractions was assayed using nitrocefin and the concentration of fusion proteins in each fraction was calculated assuming the same specific activity as the WT enzyme. Fusion proteins were purified by IMAC chromatography. The imidazole-eluted BLA-active fractions were pooled and the purity was found better than 95% as checked by SDS-PAGE. Figure 6.

Screening a peptide-BLA Scaffold Library

COSTAR plates (96-well) were coated with 0.5 μg (100 μL of 5 μg/mL) $hVEGF_{165}$ (Preprotech, Rocky Hill, NJ) with gentle rocking at 4 °C O/N, followed by blocking with Superblock blocking buffer (Pierce) for several hours at room temperature. His-tag purified samples of pCM01 and pCM04 were diluted serially into BLA assay buffer and 100 μl portions were transferred to VEGF coated wells. After one hour, plates were washed six times with PBS, 0.05% Tween 20 and 200 μL of nitrocefin assay buffer containing 0.1 mg/ml nitrocefin (Oxoid, New York) was added to measure residual bound beta-lactamase activity, Abs_{490}/min. Control wells contained pCB04 beta-lactamase as a control (Figure 7).

Inhibition of HUVEC proliferation by aVEGF peptides.

HUVE cells (Cambrex, East Rutherford, NJ) were passaged 1-5 times and maintained according to manufacturers instructions. HUVEC growth was stimulated by 0.03 to 20 ng/ml VEGF with the highest proliferation at 10 ng/ml VEGF165; this concentration was used in subsequent experiments. A series of a VEGF peptides from 0.5 nM to 25 μM (and a anti-VEGF MAb control (R&D Systems)) were mixed with 10 ng/mL VEGF prior to addition to HUVECs seeded in triplicate in 96-well plates. Cell proliferation was measured by ^{3}H-thymidine incorporation (Figure 8). Significant inhibition was observed down to 0.4 μM.

Inhibition of Blood vessel tube formation by VEGF peptide conjugates

This *in vitro* angiogenesis assay was obtained as a kit from CHEMICON (Temecula, CA) and used according to the manufacturers instructions. The assay represents a simple model of angiogenesis in which the induction or inhibition of tube formation by exogenous signals can be monitored. An endothelial cell suspension of low passage HUVE cells was mixed with different concentrations of the inhibitor in the presence of 10 ng/mL VEGF, before adding the cells to "ECMatrix". ECMatrix is a solution that is polymerized *in situ* and provides a solid gel of basement proteins prepared so that endothelial cells align and form hollow tube-like structures. Tube formation is a multi-step process involving cell adhesion, migration, differentiation and growth. The resulting tube formation was measured under an inverted light microscope at 20X-100X magnification. Significant inhibition of tubule formation was
observed above 1 μM peptide, results not shown.

Construction of phage-displayed VEGF biased peptide libraries:

The affinity maturation libraries used for panning VEGF were constructed using the C7C gene III phage-display system ((Noren & Noren, 2001)). Oligonucleotides were synthesized and phosphorylated. The oligonucleotides used to construct the libraries employ NNK (where N = G, A, T, C and K= G or T) codons. The NNK cloning scheme eliminates the potential for two stop codons and still encodes all twenty amino acids. The random peptide library displays 9 random amino acids with two cysteines fixed at positions 2 and 9 (XCX7CGGGS; X represents any amino acid).

Seven CK37282 biased peptide libraries were created using the same methods as for the random library (Noren & Noren, 2001).

Construction of aVEGF Bowman Birk Inhibitor (BBI^{VEGF}).

A synthetic gene coding for Bowman Birk Inhibitor (Figure 9; Operon) with appropriate restriction sites for introducing small peptide coding sequences into the trypsin loop (SacI-EcoRI) and/or chymotrypsin loop (EcoRI-SalI) was cloned into pET-22b (Novagen)) using NdeI/XhoI cloning sites according to standard procedures ((Sambrook,

1989) The resulting vector, pET BBI, was used as a template to insert the sequences CK37281, CK37282 into BBI loops as double-stranded oligonucleotide cassettes (Operon). Constructs were transformed into BL-21(DE3) *E coli*, and plated on 50 μg/mL Ampicillin. Plasmid DNA from individual clones was isolated (Qiagen) and the correct inserts confirmed by DNA sequencing.

Fusion proteins and (WT) were expressed in 14-L fermentors. Cell pastes were harvested and protein isolated from inclusion bodies using a modification of the FoldIt screening procedure (Hampton) (Figure 10).

BIAcore™ binding analysis: BBI^{VEGF}

Affinities of BBI VEGF constructs for VEGF were measured using BIAcore-3000 surface plasmon resonance (Biacore, Inc., Piscataway, NJ). A CM5 sensor chip was conditioned with 50 mM NaOH and activated for covalent coupling of VEGF using *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions (Biacore, Piscataway, NJ). VEGF (human $VEGF_{165}$, Biosource, Int. Camarillo, CA) was diluated to 5μg/mL in 20mM sodium acetate, pH 4.8 and injected at a flow rate of 2 μL/min to achieve approximately 1000 to 6000 response units (RU) of coupled protein. Trypsin and chymotrypsin was similarly coupled to the CM5 sensor chip to approximately 850 to 3500 RU in remaining lanes. A solution of 1M ethanolamine was injected as a blocking agent. Selective binding affinity to VEGF of refolded BBI VEGF is shown in Figure 11.

In vitro cell proliferation assay to test the activity of hVEGF inhibitory peptides.

The antiproliferative activity of VEGF inhibitory peptides was determined using human umbilical vein endothelial cells (HUVEC) (Clonetics) as follows. An early passage (less than six) of HUVEC was seeded in 96-well plates at 5000 cells per well and starved for 18 hrs in 200 μl EBM medium (Clonetics) without growth factors and supplemented with 0.5% fetal bovine serum (FBS) at 37 °C with 5% CO_2. The medium was replaced with 180 μl of growth medium containing EBM medium with 5% fetal bovine serum and 1% DMSO. 20 μl of VEGF preincubated for one hour with varying peptide concentrations (the final DMSO concentration of all the wells was 1%) were added to the wells for a final VEGF concentration of 10 ng/ml. Human VEGF antibody (R & D Systems) was used as a positive control. Cells with 0.31 to 20 ng/ml concentrations of VEGF alone in the growth medium were used to construct a standard growth curve. The cells were further incubated for 48 hrs, and the cell proliferation was measured using an MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit from Promega). 40 μl of the MTS tetrazolium solution was added to each well and after 3 and 4 hours incubation, the plates were read at 490 nM. The absorption of media alone was subtracted from all data points.

The VEGF inhibitory peptides CK37281 and CK37283 have IC50 in the micromolar range.

Repeat Insult Patch Testing (RIPT) of a VEGF peptide on human skin

Samples of aVEGF peptide CK37281 dosed at 0.5% (w/v) were formulated in a base formulation containing deionised water/butylene glycol. Approximately 0.2 mL of the formulation was applied to 200 human volunteers in a repeated insult patch test according to procedures designed by Clinical Research Laboratories, Inc. (Piscataway, NJ). This formulation did not demonstrate a potential for dermal irritation or sensitization, results not shown.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those

skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Abstract

The invention is directed to peptides and supported peptides for treating proliferative diseases. Specifically, the invention is directed to peptides and supported peptides for treating proliferative diseases of the skin, such as rosacea.

VEGF
3rd Round with C7C library
Acid eluted

```
CK37281 YNLYGWT-
CK37282 -TLWPTFW
CK37283 -NLWPHFW
CK37284 -SLWPAFW
CK37286 -APWNSHI
CK37287 -APWNLHI
CK37289 -TLWPSYW
Consensus  LWP  W
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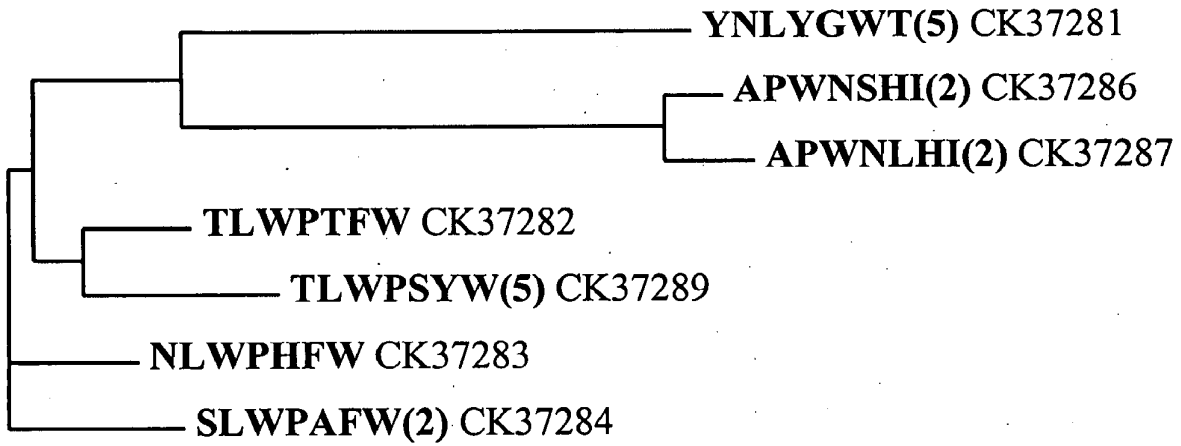
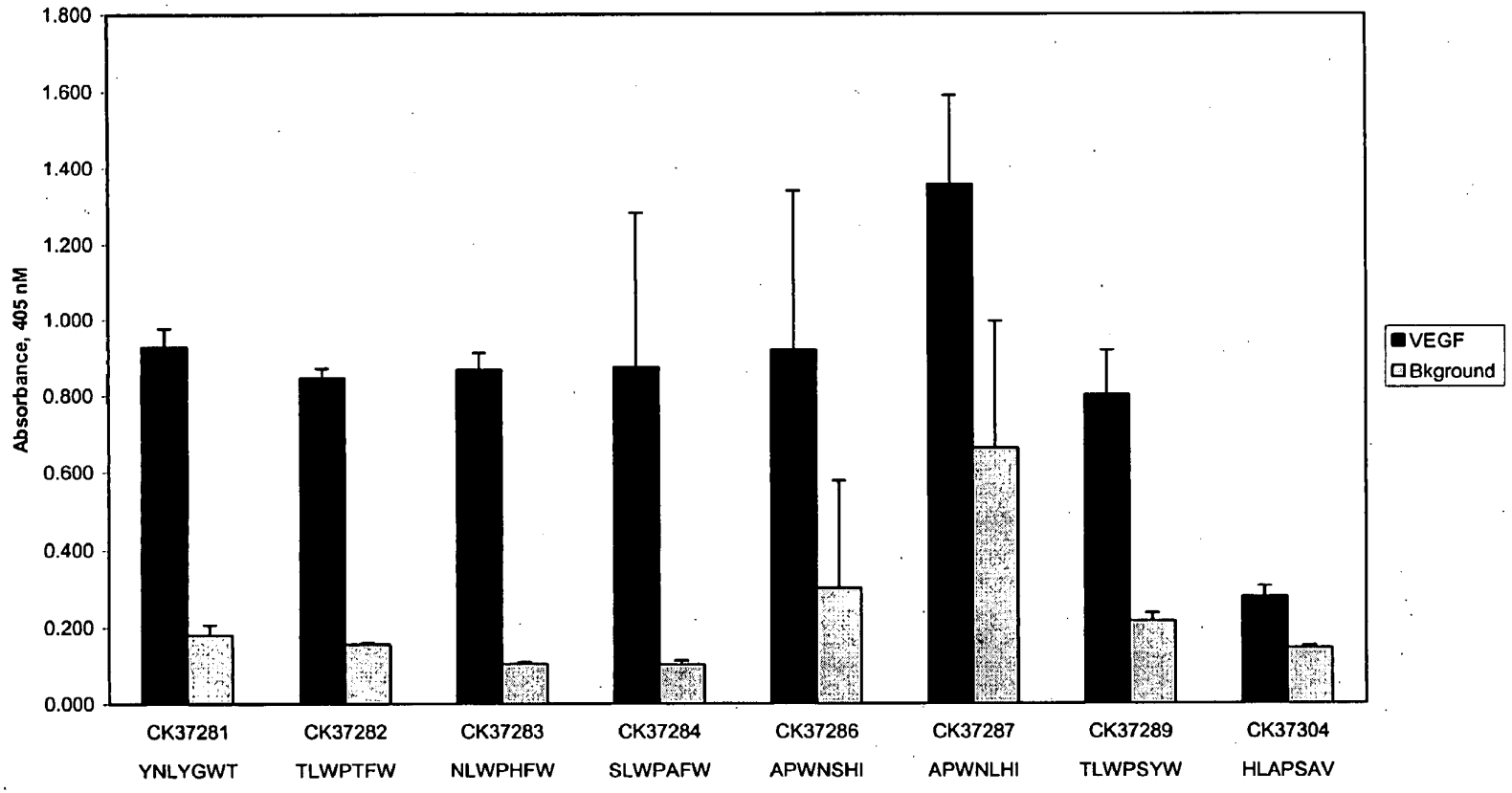


FIGURE 1

FIGURE 2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1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
VEGF
Bkground
CK37281
YNLYGWT
CK37282
TLWPTFW
CK37283
NLWPHFW
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CK37304
HLAPSAV

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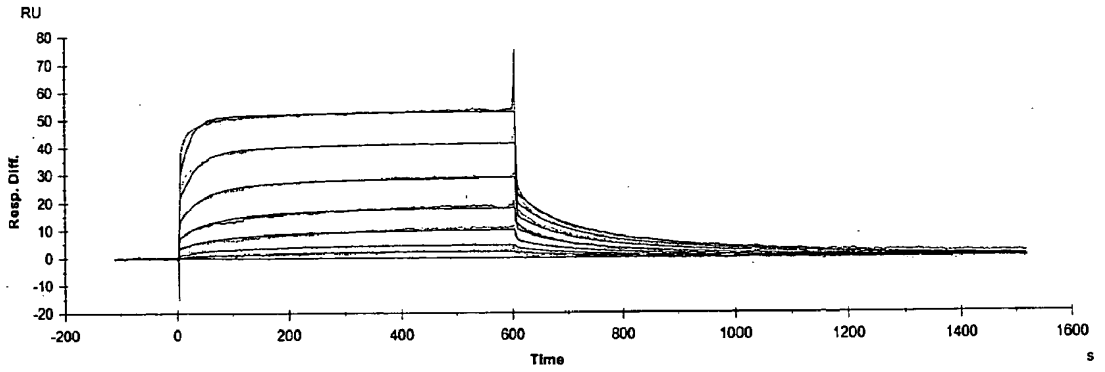

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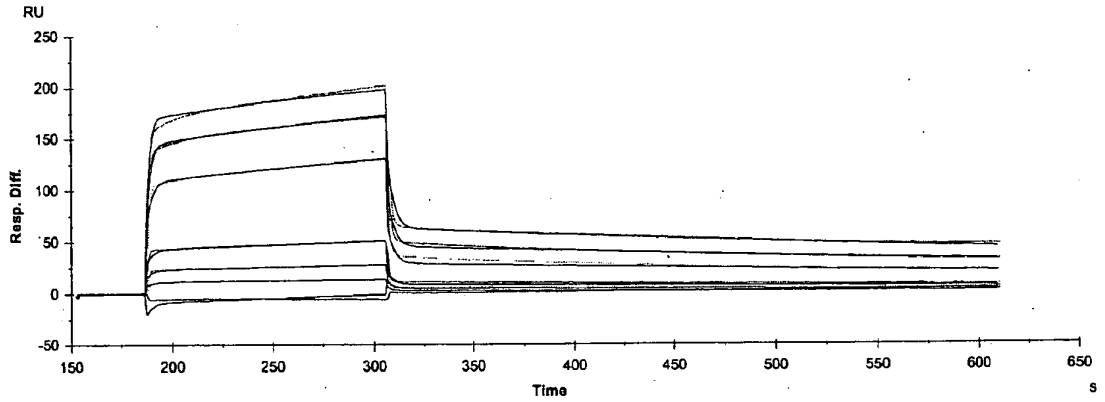

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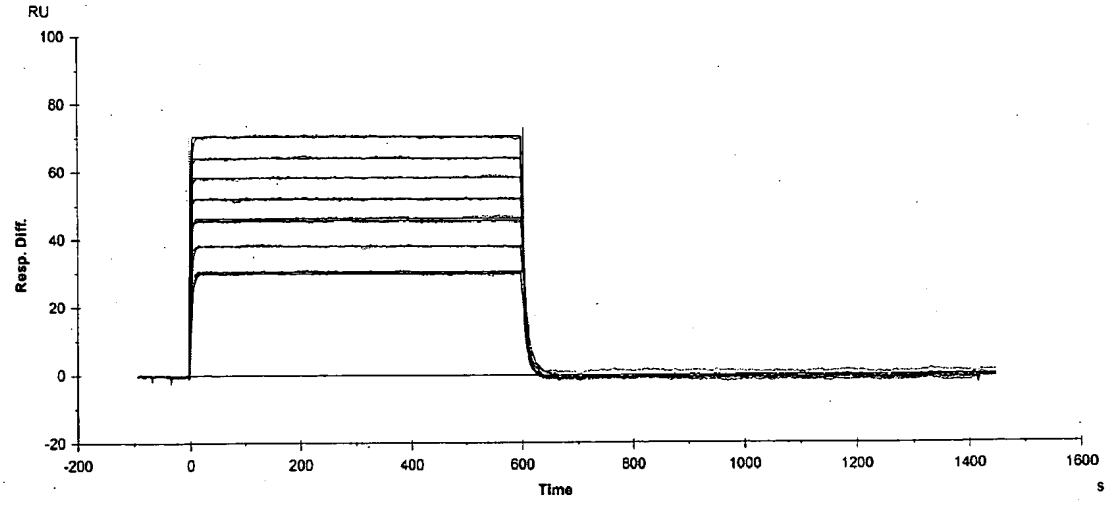
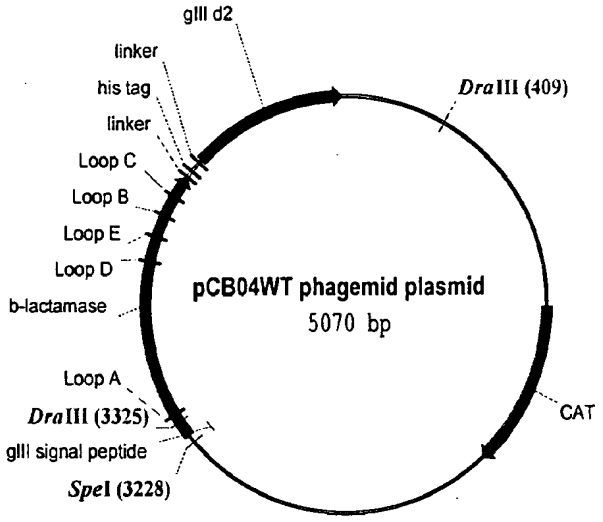
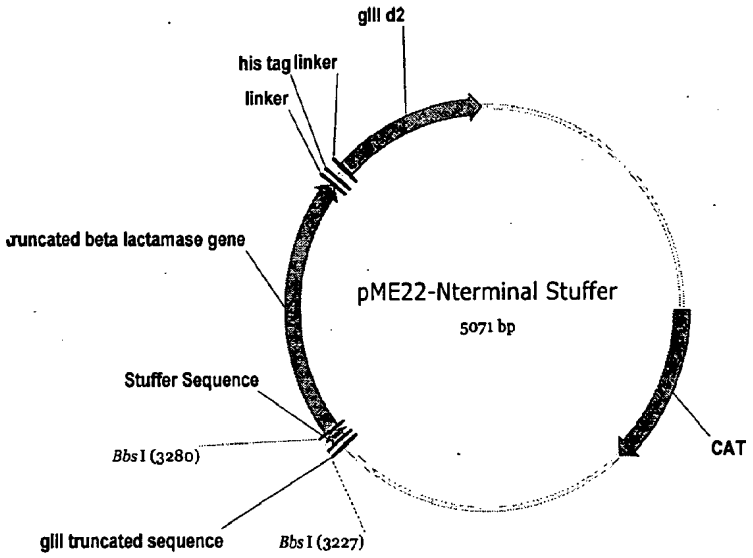


Figure 3

(A)


gIII d2
linker
his tag
linker
Loop C
Loop B
Loop E
Loop D
b-lactamase
Loop A
DraIII (3325)
gIII signal peptide
SpeI (3228)
DraIII (409)
pCB04WT phagemid plasmid
5070 bp
CAT

(B)


gIII d2
his tag linker
linker
Truncated beta lactamase gene
Stuffer Sequence
BbsI (3280)
gIII truncated sequence
BbsI (3227)
pME22-Nterminal Stuffer
5071 bp
CAT

(C)

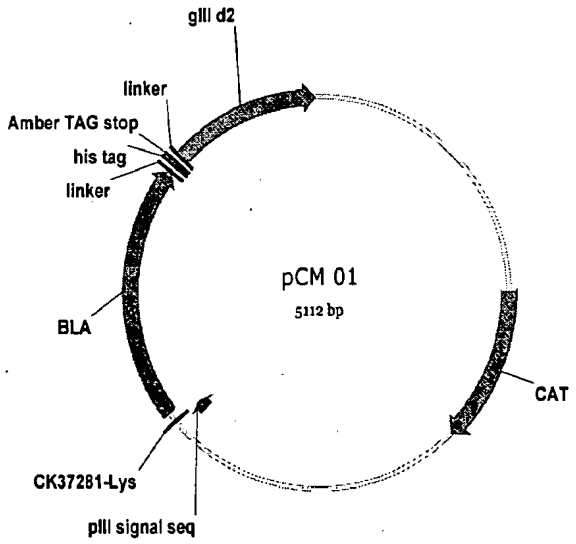

gIII d2
linker
Amber TAG stop
his tag
linker
BLA
CK37281-Lys
pIII signal seq
pCM 01
5112 bp
CAT

Figure 4

Figure 5

pIII Leader-AmpC Sequence

```
       SpeI                                                                                                DraIII
      ~~~~~~~                                                                                            ~~~~~~~~~~
IleProLeuValValProPheTyrSerHisSerThrProValSerGluLysGlnLeuAlaGluValValAlaAsnThrIleThrProLeuMetLysAlaGlnSerValPro
ATTCCACTAGTCGTTCCTTTCTATTCTCACTCTACGCCAGTGTCAGAAAAACAGCTGGCGGAGGTGGTCGCGAATACGATTACCCCGCTGATGAAAGCACAGAGTGTTCCA
TAAGGTGATCAGCAAGGAAAGATAAGAGTGAGATGCGGTCACAGTCTTTTTGTCGACCGCCTCCACCAGCGCTTATGCTAATGGGGCGACTACTTTCGTGTCTCACAAGGT
```

Digest with SpeI and DraIII (note: Second DraIII site in vector)

```
       SpeI                                                                                              DraIII
      ~~~~~~~                                                                                          ~~~~~~~~~~
IlePro      LeuValValProPheTyrSerHisSerThrProValSerGluLysGlnLeuAlaGluValValAlaAsnThrIleThrProLeuMetLysAlaGlnSerValPro
ATTCCA      CTAGTCGTTCCTTTCTATTCTCACTCTACGCCAGTGTCAGAAAAACAGCTGGCGGAGGTGGTCGCGAATACGATTACCCCGCTGATGAAAGCACAGA    GTGTTCCA
TAAGGTGATC      AGCAAGGAAAGATAAGAGTGAGATGCGGTCACAGTCTTTTTGTCGACCGCCTCCACCAGCGCTTATGCTAATGGGGCGACTACTTTCGTG    TCTCACAAGGT
```

Replace with Stuffer Fragment

```
IlePro      LeuValSerSerIleLysSerThrThrAlaCysLeuGlnIleLeuLysThrGlyGlyGlyGlyArgGluTyrAsp  <-- out of frame stuffer sequence
                          bla correct sequence frame   -->     AlaGluValValAlaAsnThrIleThrProLeuMetLysAla    GlnSerValPro
       SpeI                  SalI           PstI       BbsI                                          DraIII
     ~~~~~~~                ~~~~~~         ~~~~~~~    ~~~~~~                                        ~~~~~~~~~
ATTCCA      CTAGTGTCTTCGATCAAGTCGACAACAGCCTGTCTGCAGATCCTGAAGACTGGCGGAGGTGGTCGCGAATACGATTACCCCGCTGATGAAAGCAC    AGAGTGTTCCA
TAAGGTGATC      ACAGAAGCTAGTTCAGCTGTTGTCGGACAGACGTCTAGGACTTCTGACCGCCTCCACCAGCGCTTATGCTAATGGGGCGACTACTTTCGTGTCT    CACAAGGT
                  BbsI
                 ~~~~~~
```

Digest Stuffer Fragment with BbsI

```
IlePro                                                                                  AlaGluValValAlaAsnThrIleThrProLeuMetLysAlaGlnSerValPro
       SpeI                  SalI           PstI       BbsI                                                    DraIII
     ~~~~~~~                ~~~~~~         ~~~~~~~    ~~~~~~                                                  ~~~~~~~~~
ATTCC      ACTAGTGTCTTCGATCAAGTCGACAACAGCCTGTCTGCAGATCCTGAAGACTG      GCGGAGGTGGTCGCGAATACGATTACCCCGCTGATGAAAGCACAGAGTGTTCCA
TAAGGTGAT      CACAGAAGCTAGTTCAGCTGTTGTCGGACAGACGTCTAGGACTTCTGACCGCC      TCCACCAGCGCTTATGCTAATGGGGCGACTACTTTCGTGTCTCACAAGGT
                  BbsI
                 ~~~~~~
```

Replace with N-term Library (VegF)

```
IlePro  LeuValValProPheTyrSerHisSer AlaCysXxxXxxXxxXxxXxxXxxXxxCysGlyGlySer  ThrProValSerGluLysGlnLeu   AlaGluValValAla
ATTCC  ACTAGTCGTTCCTTTCTATTCTCACTCT GCTTGTXXXXXXXXXXXXXXXXXXXXXTGCGGTGGAGGTTCG  ACGCCAGTGTCAGAAAAACAGCTG   GCGGAGGTGGTCGCG
TAAGGTGAT  CAGCAAGGAAAGATAAGAGTGAGA CGAACAXXXXXXXXXXXXXXXXXXXXXACGCCACCTCCAAGC  TGCGGTCACAGTCTTTTTGTCGACCGCC   TCCACCAGCGC
```

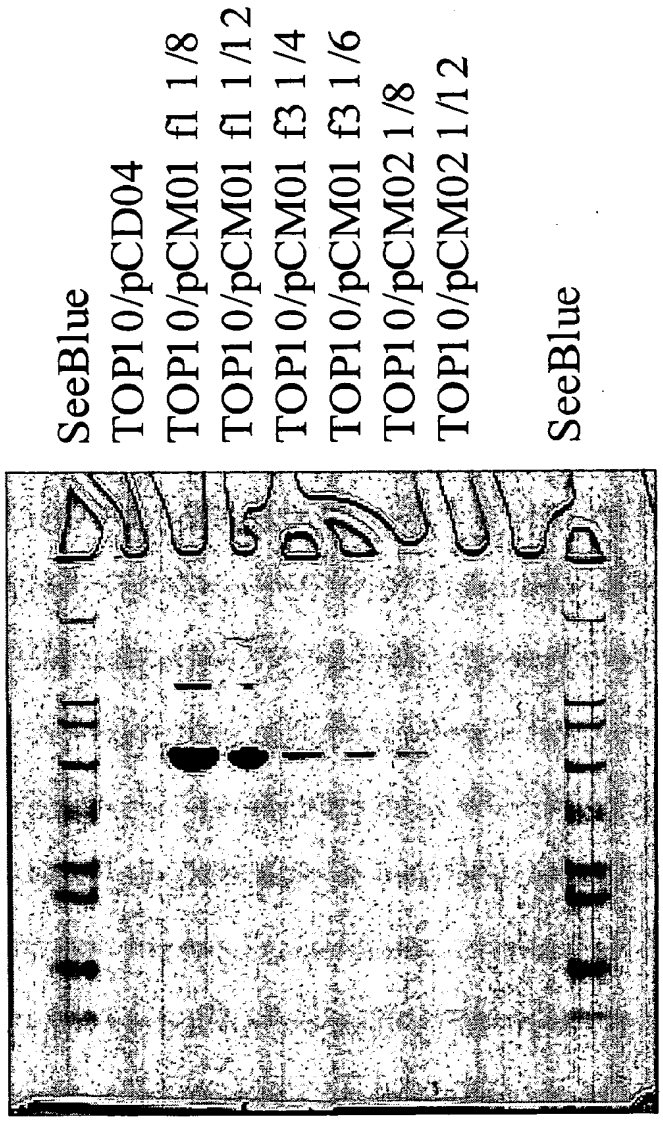

SeeBlue
TOP10/pCD04
TOP10/pCM01 f1 1/8
TOP10/pCM01 f1 1/12
TOP10/pCM01 f3 1/4
TOP10/pCM01 f3 1/6
TOP10/pCM02 1/8
TOP10/pCM02 1/12
SeeBlue

Figure 6

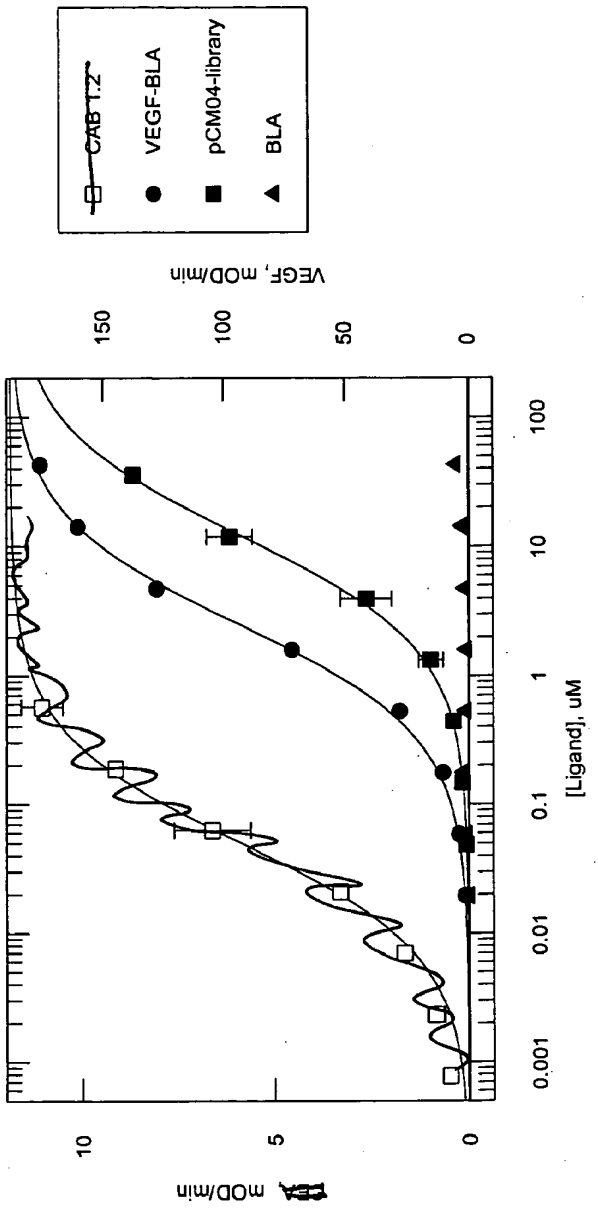

CAB 1.2
VEGF-BLA
pCM04-library
BLA
VEGF, mOD/min
150
100
50
0
10
5
0
mOD/min
0.001
0.01
0.1
1
10
100
[Ligand], uM

Figure 7

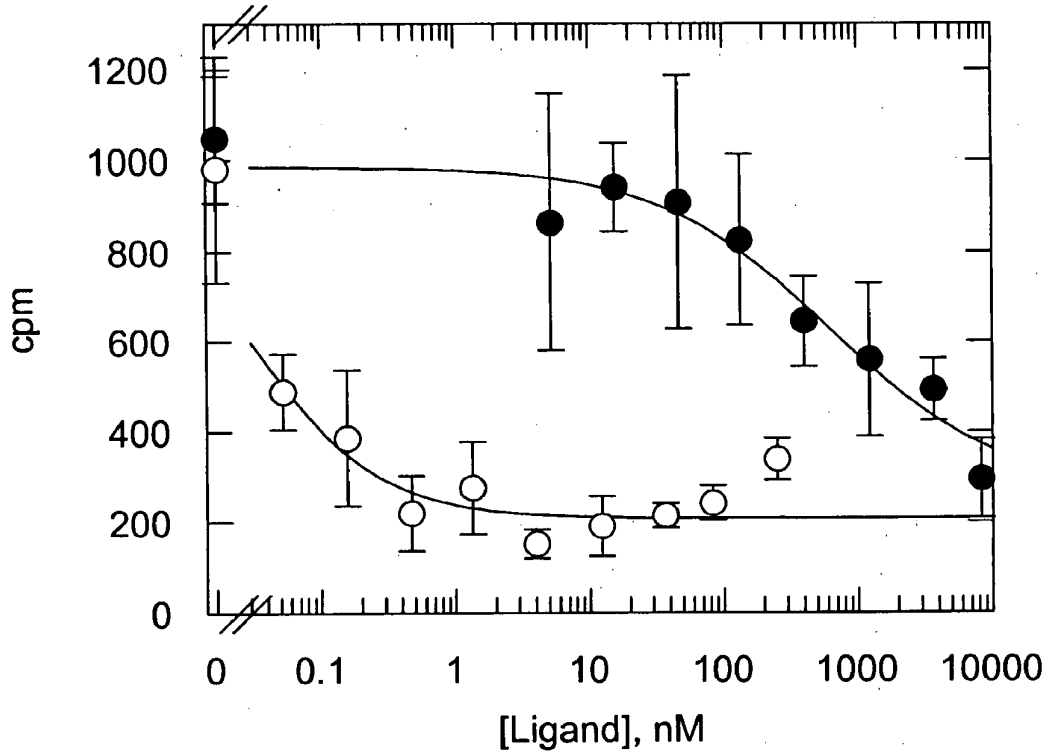

1200
1000
800
600
400
200
0
cpm
0
0.1
1
10
100
1000
10000
[Ligand], nM

Figure 8

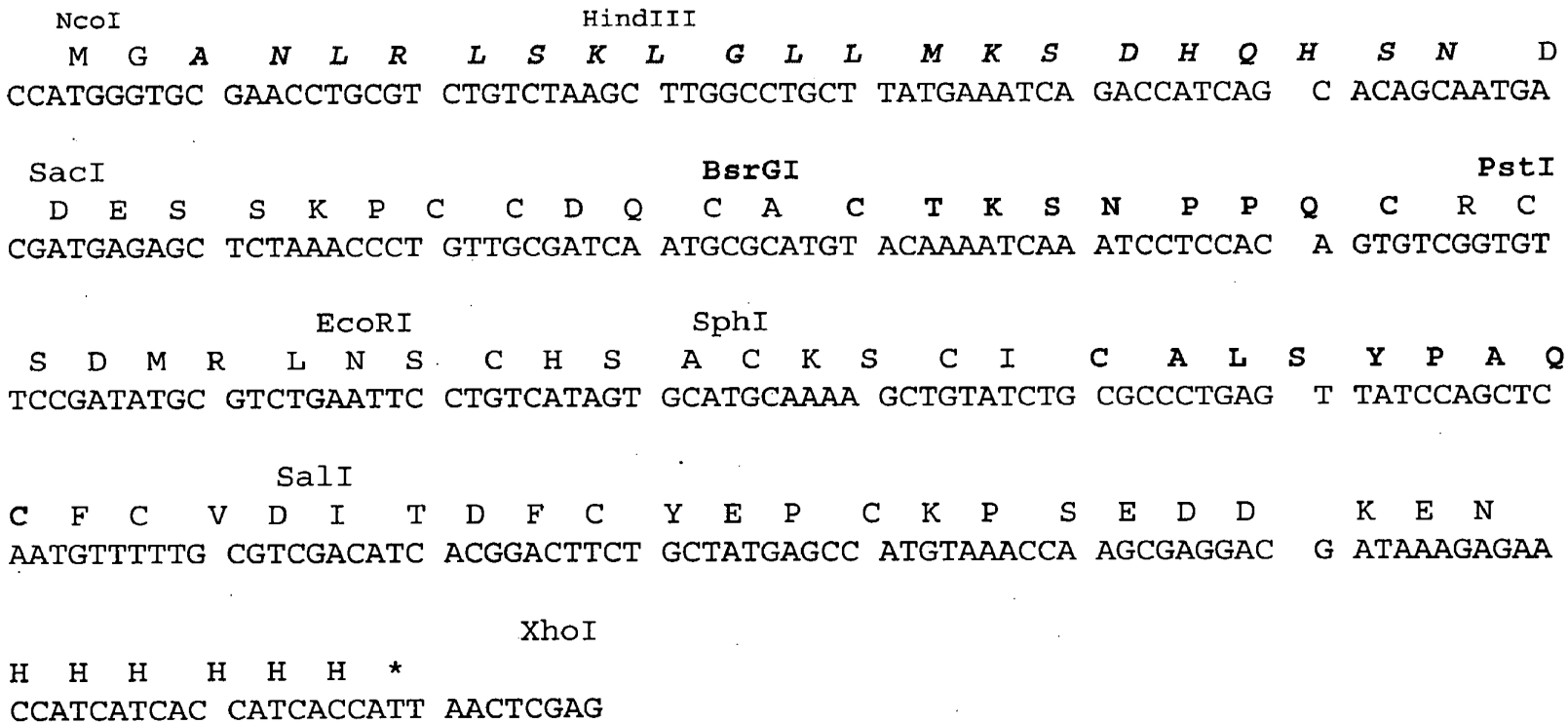

NcoI
HindIII
M G A N L R L S K L G L L M K S D H Q H S N D
CCATGGGTGC GAACCTGCGT CTGTCTAAGC TTGGCCTGCT TATGAAATCA GACCATCAG C ACAGCAATGA
SacI
BsrGI
PstI
D E S S K P C C D Q C A C T K S N P P Q C R C
CGATGAGAGC TCTAAACCCT GTTGCGATCA ATGCGCATGT ACAAAATCAA ATCCTCCAC A GTGTCGGTGT
EcoRI
SphI
S D M R L N S C H S A C K S C I C A L S Y P A Q
TCCGATATGC GTCTGAATTC CTGTCATAGT GCATGCAAAA GCTGTATCTG CGCCCTGAG T TATCCAGCTC
SalI
C F C V D I T D F C Y E P C K P S E D D K E N
AATGTTTTTG CGTCGACATC ACGGACTTCT GCTATGAGCC ATGTAAACCA AGCGAGGAC G ATAAAGAGAA
XhoI
H H H H H H *
CCATCATCAC CATCACCATT AACTCGAG

Figure 9

Figure 10

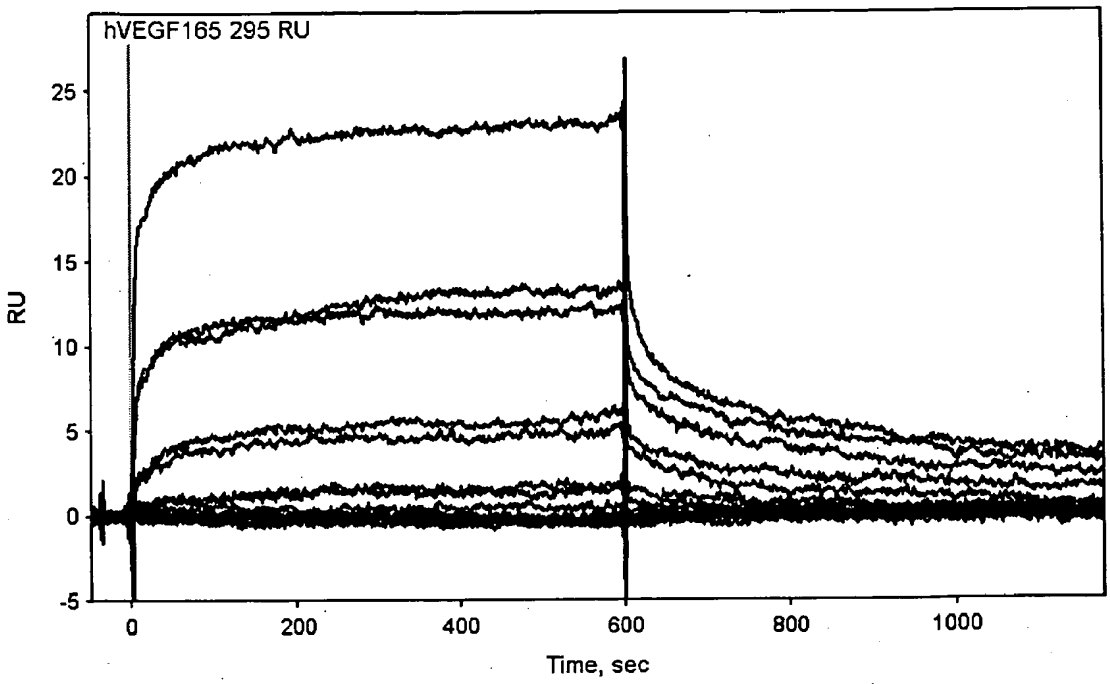

hVEGF165 295 RU
RU
25
20
15
10
5
0
-5
0
200
400
600
800
1000
Time, sec

Figure 11

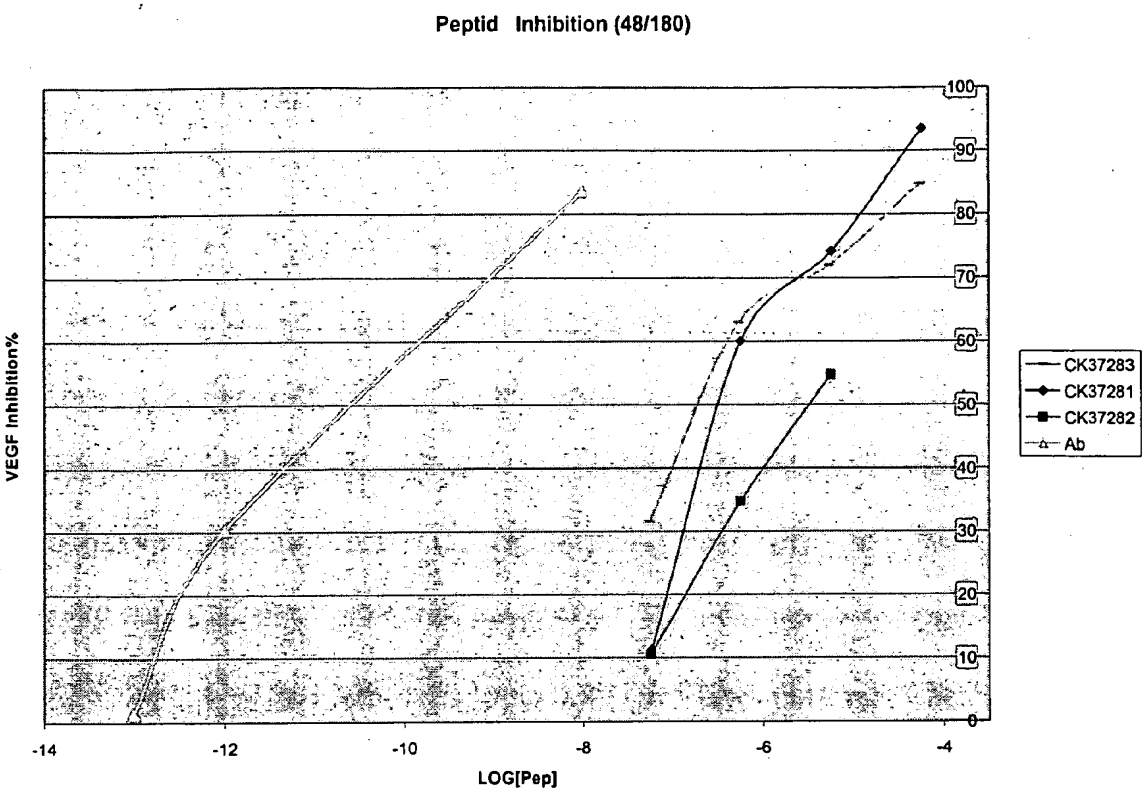

Peptid Inhibition (48/180)
VEGF Inhibition%
100
90
80
70
60
50
40
30
20
10
0
-14
-12
-10
-8
-6
-4
LOG[Pep]
CK37283
CK37281
CK37282
Ab

Figure 12

FIGURE 13

BBI-VEG1: ddesskpccdqcacynlygwtcrcsdmrlnschsackscicalsypaqcfcvditdfcyepckpseddken

BBI-VEGF2: ddesskpccdqcactksnppqcrcsdmrlnschsackscacynlygwtcfcvditdfcyepckpseddken

BBI-VEGF12: ddesskpccdqcacynlygwtcrcsdmrlnschsackscacynlygwtcfcvditdfcyepckpseddken

Figure 13